Influence of hyperthermia and antioxidant supplementation on redox balance and heat shock protein response to exercise

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INFLUENCE OF HYPERTHERMIA AND ANTIOXIDANT SUPPLEMENTATION ON REDOX BALANCE AND HEAT SHOCK PROTEIN RESPONSE TO EXERCISE

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ABSTRACT

Physical activity of moderate intensity and duration leads to healthy biological adaptations in humans. However, very intense and prolonged exercise may induce disruption in redox balance, potentially increasing oxidative stress. In addition, exposure to environmental heat stress and associated hyperthermia further increases oxidative stress and may induce the expression of heat shock proteins. However, antioxidant supplementation is believed to minimise the effect of oxidative stress and may therefore help reduce or limit the heat shock response to exercise heat stress.

The first study (**Chapter 4**) examined whether exertional heat illness (EHI) casualties among military recruits may exhibit greater disturbances in redox balance following exercise compared to non-EHI controls. Nine (n=9) recruits were identified as having suspected EHI during the Loaded March (LM) on day 1, with a peak mean (SD) body core temperature of 40.1 (0.5) °C. Fifteen (n=15) recruits were identified as having suspected EHI during the Log Race (LR) on day 2, with a peak mean (SD) body core temperature of 39.7 (0.5) °C. A further twenty-one (n=21) recruits, which successfully finished both LM and LR events, were treated as controls (CON). Interestingly, the plasma antioxidant concentration was significantly elevated from pre to post-exercise *($p<0.001$)* for EHI and CON groups, during both LM and LR events, with no changes on lipid peroxide protein carbonyl concentrations. These data suggest there is no increase in lipid peroxide or protein carbonyl level damage in response to intense hyperthermic military exercise, regardless of acute heat illness. It is possible that military training augments the body's defence capabilities, thus reducing oxidative stress and damage induced by free radical production.

Abstract

To date there is a scarcity of data examining the effects of acute intake of antioxidant supplements on oxidative stress and heat shock response during continuous exercise in a hot environment. Hence, the aims of the second study (**Chapter 5**) were to examine the effects of acute ingestion of Quercetin (Q), Quercetin + vitamin C (QC) or placebo (P) 14 hours before, 2 hours before and every 20 minutes during trials on oxidative stress and heat shock response. In this randomised, crossover study 10 recreationally active males (age 21±2 y, $\dot{V}O_2$max 54.9±8.4 ml.kg.min^{-1}) completed three running trials at 70% $\dot{V}O_2$max for 60 minutes in the heat (33.0±0.3°C; 28.5±1.8% relative humidity). Exercise heat stress significantly elevated plasma quercetin (p=0.02), antioxidant power (FRAP) (*p<0.001),* plasma heat shock protein 70 (HSP70) (*p=0.009*) and plasma heat shock protein 90α (HSP90α) (*p<0.001*) over time, but no differences were detected between trials. Also, no changes were observed in protein carbonyl concentration. Acute intake of quercetin significantly increased the level of plasma quercetin however, this did not affect the plasma antioxidant capacity or heat shock response to exercise heat stress. The increases in plasma HSP70 and HSP90α concentrations might act as supplementary antioxidants, reducing the oxidative damage reflected in the absence of changes in protein carbonyl.

Exercise heat stress is effective in inducing both intracellular HSP70 (muscle and peripheral blood mononuclear cell (PBMC)) and extracellular HSP70 (plasma) concentrations. Thus, the third study (**Chapter 6**) tested the hypothesis that this acute quercetin supplementation would induce similar trends in plasma HSP70 and intracellular HSP70 concentrations 2 days following exercise heat stress. In this randomised, crossover study, 9 recreationally active males (age 22±2y, $\dot{V}O_2$max 50.3±3.3ml.kg.min^{-1}) completed three running trials at 70% $\dot{V}O_2$max for 60 minutes in the heat (32.9±0.3°C; 28.3±1.2% relative humidity). This study demonstrated that there is no positive relationship between both intracellular of HSP70 (muscle and PBMC) and plasma HSP70 (eHSP70) 2 days

following exercise heat stress. These data suggest that the release of eHSP70 could originate from others tissue or cells. Additionally, the absence of differences between trials in the expression of muscle HSP70, PBMC HSP70 and plasma HSP70 might indicate it is implausible that quercetin might inhibits the expression of HSP70 in plasma, muscle and PBMC 2 days following the exercise heat stress stimulus.

Overall, the results from this thesis emphasise that the hyperthermia experienced in response to exercise and environmental heat stress could potentially influence the human redox response and heat shock response. Besides, there is reasonable evidence that acute quercetin co-ingestion with vitamin C has the potential to improve the bioavailability and bioactive effects of quercetin, however, the effects of quercetin supplementation in reducing oxidative stress in response to exercise heat stress remains to be elucidated. In addition, the anti-oxidative ability of acute ingestion of quercetin to suppress the intracellular and extracellular heat shock response remains uncertain and worthy for further investigation.

Key words: antioxidant supplements, quercetin, oxidative stress, heat shock response, heat, hyperthermia, exercise.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
beats.min^{-1}	beats per minute
BMI	body mass index
CAT	catalase
CK	creatine kinase
cm	centimetre
CV	cardiovascular
DNA	deoxyribonucleic acid
DNPH	2,4- dinitrophenylhyrdazine
FR	free radical
FRAP	ferric reducing ability of plasma
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GXT	graded exercise test
H_2O_2	hydrogen peroxide
hb	haemoglobin
hct	haematocrit
HOCl	hypochlorous acid
kDa	kiloDalton
kg	kilogram
LOOH	lipid hydroperoxides
MAPK	mitogen activated protein kinase
Mb	myoglobin
MDA	malondialdehyde
metHb	methaemoglobin
metMb	metmyoglobin
MeOH	methanol
mM	milimolar
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
nM	nanomolar
nmol/mg	nanomolar per miligram
$NO^{\bullet}$	nitric oxide
$NO_2^{\bullet}$	nitrogen dioxide
$O_2^{\bullet -}$	superoxide anion

O_3	ozone
$OH^{\bullet}$	hydroxyl radical
$ONOO^{-}$	peroxynitrite
ORAC	oxygen radical absorbance capacity
oxyHb	oxyhaemoglobin
oxyMb	oxymyoglobin
PBMC	peripheral blood mononuclear cell
PC	protein carbonyl
QQ	oquinone/ quinonmethide
$RO^{\bullet}$	alkoxyl
$ROO^{\bullet}$	peroxyl
$ROOH^{\bullet}$	hydroperoxyl
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TAC	total antioxidant capacity
TBARS	thiobarbituric reactive substances
TEAC	trolox equivalent antioxidant capacity
UA	uric acid
UV	ultraviolet
$\dot{V}O_2max$	maximum rate of oxygen uptake
XD	xanthine dehydrogenase
XO	xanthine oxidase
ºC	degree Celcius
ug/L	microgram per litre
ug/mL	microgram per microlitre
µl	microlitre
µM	micromolar
$^1O_2$	singlet oxygen
%	percent

CHAPTER 1

GENERAL INTRODUCTION

Physical activity is arguably one of the most important components of healthy lifestyle. It can increase longevity and quality of life (Bouchard & Shephard, 1994). Regular physical activity has countless health benefits, delaying all-cause mortality and decreasing the risk of major illnesses such as cardiovascular disease, cancer, and diabetes. In contrast, sedentary habits are associated with an increased risk of all-cause mortality (Blair et al. 2001; Crespo et al. 2002; Oguma et al. 2002). Based on extensive evidence (Paffenbarger et al., 1986; Pate et al. 1995), physical activity guidelines generally recommend that individuals should engage in at least 30 minutes per day of moderate-vigorous intensity exercise, to improve and maintain health.

Heat production increases progressively as exercise intensity increases, but it is constant if the exercise intensity is constant. Core temperature rises in a linear manner and plateau as the thermal steady-state is achieved (i.e. heat dissipation equals metabolic heat production). However, core body temperature may not plateau during intense exercise, particularly in hot/humid environments, where metabolic heat production is greater than the capacity for heat dissipation (González-Alonso et al., 2000). Heat is transferred from the contracting muscles to the normally less mobile body trunk and to the skin surrounding the exercising limbs then about 80% of heat dissipate through evaporation of sweat (González-Alonso et al., 2000). However, when intense exercise occurs in the hot environment, it will challenge the cardiovascular (CV) system to meet the maximal demands of the muscle and the skin simultaneously (Casa, 1999; González-Alonso et al., 2008; Rowell, 2011). Elevation of body temperature increased the cutaneous blood flow demands in order to transfer the metabolic heat from the core to the skin (Kenney & Johnson, 1992). This demand competes with the metabolic demands due to increase of

blood circulation to the active muscle during exercise (Gonzalez-Alonso et al., 2008). Therefore, exercise and/or body exposure to a warmer environment could disturb the heat equilibrium due to the deviation in circulatory and thermoregulatory systems, leading to hyperthermia (elevated body temperature).

Exercise in a hot environment, which leads to an increase in core body temperature (hyperthermia) could be a supplementary factor that induces oxidative stress to DNA, proteins and lipids (Bruskov et al., 2002; Grasso et al., 2003; Zhao et al., 2006). Furthermore, there is a plausible association between oxidative stress and heat-related illnesses, with studies proposing that oxidative stress could be a crucial adverse factor in boosting the severity of heat illnesses including heat syncope, heat exhaustion, heat cramps and heat stroke (Adachi et al., 2009). Several studies have demonstrated that heat stress could enhance oxidative stress in humans during exercise (Laitano et al., 2010; McAnulty et al., 2005; Morton et al., 2007; Ohtsuka et al, 1994; Sureda et al., 2015). Cells exposed to hyperthermia produce reactive oxygen species such as superoxide anion ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) (Belhadj Slimen et al., 2014; Davidson & Schiestl, 2001; Flanagan et al., 1998; Katschinski et al., 2000).

Oxidative stress occurs when there is excessive production of free radicals (FR) known as unstable molecules, which overwhelm antioxidant defences, disrupting redox balance and causing damage to cells, including skeletal muscle and vital organs (Sies 1985; Jones 2006; Sies & Jones 2007; Kassahn et al. 2009; Sies 2015). The human body is equipped with immensely efficient antioxidant defence systems. Antioxidants are the substances that can scavenge free radicals and help to decrease the magnitude of oxidative stress induced damage. These consist of nonenzymatic (e.g glutathione, uric acid, coenzyme Q, etc.), enzymatic (e.g superoxide dismutase (SOD), glutathione peroxidase (GPX),

glutathione reductase, catalase etc.) and dietary antioxidants (e.g tocopherols (vitamin E), carotenoids (b-carotene), ascorbic acid (vitamin C), flavonoids and etc.

Further cellular protection is provided by heat shock proteins (HSPs) that exist in both intracellular and extracellular spaces, to aid in response to heat stress, oxidative stress or other forms of cellular damage (Kalmar & Greensmith, 2009). Due to the increased HSP concentrations induced by oxidative stress, these proteins are also reported to have an antioxidant effect (Fehrenbach & Northoff, 2001). Intracellular HSPs are the family of stress response proteins involved with multiple cytoprotective functions, including molecular chaperones that play a role in inhibiting the aggregation of folded protein. These chaperones assist with correct protein refolding and transferring proteins safely to the correct compartment (Lancaster & Febbraio 2007; Morton et al. 2006; Ghazanfarp & Talebi 2013). While, extracellular HSPs (eHSPs) have been suggested as a form of danger signal or cellular messenger in response to the stress, injury, infection and cell damage. This can activate the innate immune response to protect the cell from subsequent insults (Borges et al., 2012; De Maio, 2011; Jolesch et al., 2012).

During exercise, the degree of oxidative damage is not only influenced by the extent of free radical production, but also the capacity of antioxidant defence systems. This is despite the fact that the body has its own intricate antioxidant defence system, which depends on endogenous production of antioxidant compounds as well as dietary intake of vitamins and minerals (Valko et al., 2007). Antioxidant supplementation may aid in protecting from cellular oxidative damage by maintaining the redox balance and assisting in recovery by boosting the immune function after intense exercise, thus improving athletic performance.

It is debatable whether the body's natural antioxidant defense systems are sufficient to counterbalance the increase in FR with exercise or whether additional supplements are required. Interestingly, recent studies have demonstrated that FR act as signalling molecules to stimulate antioxidant enzyme synthesis during exercise, therefore leading to favourable exercise induced adaptations (Ji et al., 2006; Radak et. al, 2014). It was therefore hypothesized that antioxidant supplementation could hamper this adaptations (Gomez-Cabrera et al., 2009). However, not all investigations have revealed that antioxidant supplementation hampers exercise-induced activation of redox sensitive signalling pathways (Petersen et al., 2012). There is a plethora of research that has demonstrated the beneficial effects of antioxidant supplementation that show positive outcomes in exercise studies. Therefore, it could be more beneficial to only consume the antioxidant supplementation during periods of elevated exercise or hyperthermic stress.

The research described in this thesis will aim to answer the following research questions:-

1) Do military recruits that suffer an exertional heat illness (EHI) have greater levels of oxidative stress compared to non-EHI controls? (**Study 1, Chapter 4**).

2) Would the acute antioxidant supplementation minimise the effects of oxidative stress and reduce the heat shock response during exercise heat stress (**Study 2, Chapter 5**)

3) Would the acute antioxidant supplementation induce similar trends in extracellular heat shock response that are seen in the intracellular heat shock response after exercise heat stress (**Study 3, Chapter 6**).

CHAPTER 2

LITERATURE REVIEW

From the research conducted to explore the redox homeostasis of exercise in the past three decades, it is now clear that acute and chronic physical activity have different effects on oxidative stress; studies have demonstrated that acute exercise induces free radical (FR) production that leads to oxidative stress, but regular exercise training (chronic) induces the endogenous antioxidant defence and protects the body against adverse effects of oxidative stress. Historically, the relevant literature has mainly focused on the adverse effects of FR, however, recent studies have proposed that exercise-induced FR production could induce some health-promoting effects of exercise. Despite previous research efforts, it is still unclear whether and how ingested exogenous antioxidants as a non-invasive strategy affect *in vivo* redox homeostasis and exercise performance.

This chapter will critically review the definition of oxidative stress, mechanisms of oxidative stress during exercise and their biological effects in humans. This will be followed by a review of how exercise, heat stress and associated hyperthermia affect redox homeostasis. Emphasis will be placed mainly on heat shock proteins, which are intrinsically linked with exercise, oxidative stress and a hot environment. This chapter will also review the endogenous antioxidant defence (enzymatic and non-enzymatic) in humans and how the exogenous supplementation of antioxidants can affect redox homeostasis during exercise.

2. OXIDATIVE STRESS, REDOX HOMEOSTASIS AND EXERCISE

Regular physical activity is one of the vital components of a healthy lifestyle (Brach et al., 2004; Nelson et al., 2007; Oja & Titze, 2011; Reiner et al., 2013). Regular physical activity has countless health benefits, delaying all-cause mortality as well as reducing the risk of major illnesses such as cardiovascular disease, cancer and diabetes. On the contrary, sedentary habits are associated with an increased threat of all-cause mortality (Blair et al., 2001; Crespo et al., 2002; Oguma et al., 2002; Pate et al.,1995). However, exercise could also produce an imbalance between FR and antioxidants, which is referred to as oxidative stress. Later, this chapter will explain thoroughly the definition of oxidative stress and how the definition has evolved over time.

During basal metabolism and even at moderate intensities of exercise, the human body produces unstable molecules known as FR, which act as valuable signaling molecules for bodily functions (Dröge, 2002). Halliwell (2007) defined FR as any chemical species known as molecules or molecular fragments comprising unpaired electrons in their molecular orbital.

Most researchers acknowledge that moderate exercise yields FR in healthy amounts leading to healthy adaptations. However, intense exercise may cause excessive production of FR, which can overwhelm the antioxidant defence and cause damage to cells, muscles and vital organs. The negative effects of FR induced by exercise could be due to an excessive level of FR production, the duration to the stress exposure, the cellular origin of FR produced and/or environmental factors (Limón-Pacheco & Gonsebatt, 2009; Mason & Wadley, 2014).

The first discovery that physical exercise can lead to an increase in lipid peroxidation appeared in 1978, Dillard and colleagues observed a 1.8-fold increase in exhaled pentane levels (biomarker of lipid peroxidation) after one hour of exercise at 75%

$\dot{V}O_2$max when compared to the production at pre and post exercise resting levels (Dillard et al., 1978). Since then, an increasing collection of studies using both animal and human subjects has accumulated to support the hypothesis that physical exercise has the potential to increase FR production and may lead to the oxidation of several biological molecules (i.e. lipids, proteins, nucleic acids) (Groussard et al. 2003; Watson et al. 2005; Powers & Jackson 2008; Fisher-Wellman & Bloomer 2009; Powers et al. 2011). In **subchapter 2.2**, this will be discussed in detail in relation to the mechanisms involved in increasing free radicals production with exercise.

There are several definitions of oxidative stress. In 1985, Sies firstly defined oxidative stress as a disruption in the oxidant and antioxidant balance on the side of the oxidants, resulting in potential damage. Oxidative stress also can be defined as an inequality between the production of FR and the capability of the human body defence system (antioxidant) to offset their harmful effects, which may associate with tissue injury (Halliwell, 2007). In addition, Kassahn et al. (2009) defined oxidative stress to be a displacement from homeostasis causing injury to a biological system. Therefore, it can be concluded that the terms ‘disturbance’, ‘disruption’ and ‘damage’ that are contained in the definition of oxidative stress implies disruption of normal function.

However, the definition of oxidative stress has evolved as the scientific understanding of the role of FR (positive and negative) in cellular physiology has expanded. In 2006, Jones redefined the definition of oxidative stress by (Sies, 1985), oxidative stress as a disruption of redox signaling and control. This was based on considerable evidence that the reactive oxygen species (ROS) and reactive nitrogen species (RNS) function in redox signaling at very low concentrations in cells and are difficult to measure directly. Therefore, a year later, the two definitions were merged by the two authors as ‘a disturbance in the pro-oxidant/antioxidant balance in favor of the oxidants, leading to a

disruption of redox signaling and control and/or molecular damage' (Sies & Jones, 2007)

A few years later, Nikolaidis et al. (2012) suggested an alternative definition of oxidative stress as 'alterations in redox homeostasis'. This definition is proposed on the basis that it is difficult to apply the term 'disruption', 'disturbance' and 'damage' in some studies, therefore the term "alteration" would be more suitable known that there could be two opposite directional changes across a wide range of biomarkers in different cellular compartments. Therefore, in this thesis, the term oxidative stress would be used when it is obvious that an increase in cellular oxidative stress has occurred with one directional change in redox biomarkers. Otherwise, any change (increase or decrease) in the level of reactive species, redox biomarkers and/or antioxidants will be referred to the alteration in redox homeostasis (Nikolaidis et al. 2012).

Three decades has passed since the first definition of oxidative stress was introduced (Sies, 1985) and yet there is no accepted classification of oxidative stress. Recently, Lushchak (2014) proposed four interesting classifications (**Table 2.1**) of oxidative stress based on intensity: basal oxidative stress (BOS), low intensity oxidative stress (LOS), intermediate intensity oxidative stress (IOS) and high intensity oxidative stress (HOS). Another interesting classification could differentiate oxidative stress into three categories: mild oxidative stress (MOS), temperate oxidative stress (TOS), and severe (strong) oxidative stress (SOS) (Lushchak, 2014). However, this proposed approach is not simple to routinely apply due to the presence of different compounds and processes involved, which could affect the responses of the systems (cells, tissues, organ and etc.) based on the inducers of the stress. Nevertheless, it could help researchers to describe the behaviour of the systems in response to different intensities of oxidative stress.

Table 2.1 Oxidative stress: definition, specific forms and classification according to intensity. Adapted from Sies (2015).

Category	Term	Author
Original definition	"A disturbance in the prooxidant and antioxidant balance in favor of the former."	Sies, 1985
Updated definition	"An imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage"	Sies & Jones, 2007
Specific form	Nutritional oxidative stress Dietary oxidative stress Postprandial oxidative stress Physiological oxidative stress Photooxidative stress • Ultraviolet (UV-A, UV-B) • Infrared-A Radiation-induced oxidative stress Nitrosative stress Reductive stress	Sies & Jones, 2007
Related terms	Oxidant stress, Pro-oxidant stress Oxidative stress status (OSS)	
Classification	Basal oxidative stress (BOS) Low intensity oxidative stress (LOS) Intermediate intensity oxidative stress (IOS) High intensity oxidative stress (HOS)	Lushchak, 2014

2.1 FREE RADICALS

Over a century ago, Gomberg (1900) discovered the existence of triphenylmethyl radical (Ph3C•), triggering a broader interest in FR. Free radicals can be defined as any chemical species that can exist independently, occupying one or more unpaired electron, being one and alone in an orbit (Aruoma, 1998; Clarkson & Thompson, 2000). Although their reactivity varies, radicals are generally less stable than non-radicals.

These substances are highly reactive with other molecules in order to gain an electron to stabilize the unpaired electron (Pham-Huy et al., 2008). The simplest free radical is an atom of the element hydrogen, which comprises a single unpaired electron and one proton, represented by the insertion of the radical dot (•) to indicate that one or more unpaired electrons exist (Aruoma, 1998).

Free radicals may be produced from many elements. Most radicals originate from reactive oxygen species (ROS) or reactive nitrogen species. ROS consist of oxygen-based free radicals, e.g. superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and hydroperoxyl ($ROOH^{\bullet}$) (**Table 2.2**) (Cooper et al., 2002). Reactive nitrogen species comprise nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) as well as the potent oxidant peroxynitrite ($ONOO^-$) (**Table 2.2**) (Cooper et al., 2002).

They have very short lifetime (from milliseconds to nanoseconds) (**Table 2.2**). FR can form new radicals once reacting with other radicals or molecules. Hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and ozone are not FR but can simply induce FR reactions in living organisms. Both the radicals as well as the FR species created via interaction with other radicals are collectively referred as reactive oxygen/nitrogen species (RONS) (Valko et al., 2007).

Table 2.2 Classification and main effects of free radicals. Adapted from Finaud et al. (2006).

Free Radical	Contraction	Half-life	Main effects
Reactive oxygen species (ROS)			
Superoxide ion	$O_2^{\bullet -}$	10^{-5} sec	Lipid oxidation and peroxidation Protein oxidation DNA damage
Ozone	O_3	Stable	
Singlet oxygen	$^1O_2$	1 µsec	
Hydroxyl radical	$OH^{\bullet}$	10^{-9} sec	
Hydrogen peroxide	H_2O_2	Stable	
Hypochlorous acid	HOCl	Stable	
Alkoxyl radical	$RO^{\bullet}$	10^{-6} sec	
Peroxyl radical	$ROO^{\bullet}$	7 sec	
Hydroperoxyl radical	$ROOH^{\bullet}$		
Reactive nitrogen species (RNS)			
Nitric oxide	$NO^{\bullet}$		Lipid peroxidation DNA damage Protein oxidation
Nitric dioxide	$NO_2^{\bullet}$	1-10 sec	
Peroxynitrite	$ONOO^{\bullet -}$	0.05^{-1} sec	
Reactive sulphur species (RSS)			
Thyll radical	$RS^{\bullet}$		Proteins oxidation DNA damage ROS production

2.2 MECHANISMS OF INCREASED FREE RADICAL PRODUCTION WITH EXERCISE

The experiment by Dillard et al. (1978) revealed that physical exercise could lead to an increase in lipid peroxidation. They observed an increase in exhaled pentane levels, which is an oxidative lipid damage by-product after cycling 60 min of 75% $\dot{V}O_2$max. A year after, (McCord, 1979) estimated that one FR is produced for every twenty-five

oxygen molecules reduced by normal respiration. After a few years, in 1982, Davies et al. confirmed this finding by determining a two- to three-fold increase in FR concentrations of muscle and liver following exercise to exhaustion. Furthermore, human studies (Bailey et al., 2007; Bailey et al., 2004) confirmed the previous animal study (Davies et al., 1982) as they discovered increased FR efflux from exercising human muscle using electron paramagnetic resonance spectroscopy.

At rest superoxide anions ($O_2^{\bullet-}$) and nitric oxide ($NO^{\bullet}$) are produced by skeletal muscle at a low rate. However, this rate is increased excessively during exercise. Aerobic exercise is typically correlates with an increment in oxygen uptake by the contracting muscle group. The rate of whole body oxygen consumption increases 10 to15-fold and the rate of oxygen consumption in active muscles during whole-body aerobic exercise can be increased more than 100-fold (Sen, 1995). The muscle groups involved, contraction modes, exercise intensity, exercise duration and exercising population could all influence the rate of oxidative stress. The potential mechanisms of increased free-radical production during exercise are as follows: -

2.2.1 Electron Leaking at the Mitochondrial Electron Transport Chain

About four decades ago, Boveris et al. (1972) found that, under resting conditions, about 2% of total oxygen uptake is converted to superoxide radicals due to insufficient coupling of electron transfer between the complex II and III. This outcome interpreted that a notable increase in FR generation would be expected during exercise because oxygen flux via active muscle may increase approximately 100 times compared to resting values to meet increased energy demands. The production of FR that occurs during contractile activity in muscle fibers is correlated to the elevation of oxygen consumption that occurs through mitochondrial respiration. This suggests an increase in production of superoxide by skeletal muscle during aerobic contractions.

However, a year later, this notion changed as Boveris & Chance (1973) discovered that leaking of FR only occurs during State 4 respiration which happens with low oxygen uptake and adenosine triphosphate (ATP) production but high membrane potential. This leaking phenomena did not occur during State 3 respiration which involves high oxygen uptake and high ATP production but low membrane potential. This may indicate that the belief mitochondrial $O_2^{\bullet -}$ production peaks during aerobic exercise is not completely accurate (Sachdev & Davies, 2008).

More recent research indicates that the main sites of mitochondrial superoxide production are complexes I and III of the electron transport chain (Barja, 1999; Muller et al., 2004). This theory illustrated that there is an expected massively increased production of FR due to electrons leaking in the mitochondrial respiratory chain of the contracting muscle cells during exercise. This corresponds to the insufficient coupling of electron transfer between the complexes I and III (**Figure 2.1**) (Vollaard et al., 2005). It seems that Complex I, which is the main site of electron leakage, releases the superoxide anion towards the mitochondrial matrix only, while Complex III appears to release superoxide anion into the matrix and outside the inner membrane (Muller et al., 2004).

The discovery of Davies et al. (1982) triggered a thought that mitochondria were the major site of superoxide generation for more than a century. Based on evidence generated in the 1970s (Boveris & Chance, 1973; Loschen et al.,1974), most studies have acknowledged a range of 2–5 % of mitochondrial oxygen consumption forming superoxide. However, St-Pierre et al. (2002) findings opposed this estimation and indicate that the upper estimate of the proportion of the electron flow that gives rise to ROS might be approximately 0.15 %.

In 2011, Wei et al. developed a novel method using mitochondrial superoxide flashes (mSOFs) that allow revealing of superoxide production in the mitochondrial matrix. Results demonstrate that the activity of mSOF increased in mitochondria during muscle contraction and is dependent on the activity of the electron transport chain. In contrast, study of isometric exercise where the oxygen pressure (PO_2) was low in the mitochondria still established an increase in the oxidative stress (Alessio, 1993). Bailey et al. (2004) also verified that free radical production through contracting muscle was correlated with decreases in intracellular PO_2 instead of increased in oxygen flux condition. These studies reinforce that it is unlikely the mainly source of FR production during exercise is because of increase in mitochondrial oxygen flux (Vollaard et al., 2005). Thus, the next subchapter will elaborate more on extramitochondrial sources of FR during exercise.

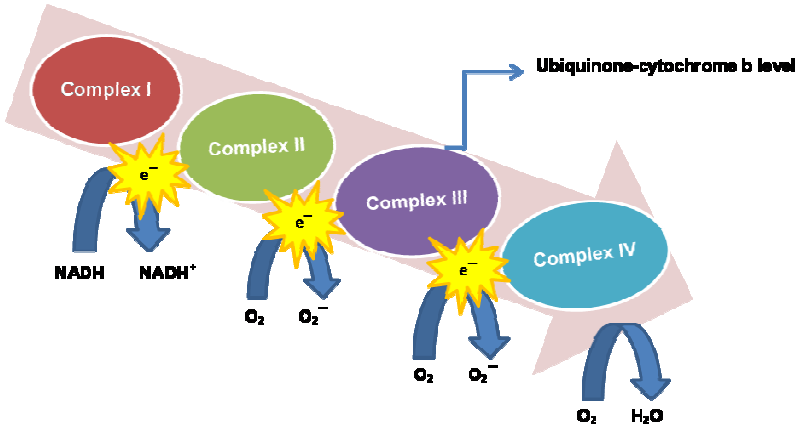


Figure 2.1 The mitochondrial respiratory chain. Electrons are transferred from complexes I, II, and III to IV. However, inadequate coupling of electron transfer can cause leakage, generating superoxide anions at different complex levels. Adapted from Gomes et al. (2012).

2.2.2 Ischemia Reperfusion

Another suggested mechanism of FR production during exercise is the ischemia-reperfusion phenomenon. During intense exercise, blood flow is shifted from many other organs and tissues to the active skeletal muscles; as a consequence other

tissues and organs (e.g. kidneys and splanchnic region) would be in a hypoxic condition (Di Meo & Venditti, 2001). Ischemia reperfusion occurs when the exercise ceases, these hypoxic tissues obtain an excessive quantity of oxygen. These would activate the conversion of the enzyme xanthine dehydrogenase (XD) to xanthine oxidase (XO) (**Figure 2.2**) (Nishino et al., 2005; Rasmussen et al., 2000). Hellsten et al. (1988) were the first to show that XO increased with chronic exercise and indicated that XO might be a significant source of ROS production during exercise.

Temporary hypoxic conditions in particular regions of the body during exercise lead to conversion of ATP to ADP, AMP, IMP, inosine and finally hypoxanthine (Norman et al., 1987; Sachdev & Davies, 2008). After the tissues are reoxygenated, degradation of hypoxanthine into xanthine and subsequently into uric acid would produce $O_2^{\bullet-}$ and H_2O_2 as by-products (**Figure 2.2**) (Gomes et al., 2012).

Xanthine dehydrogenase has an important role in the formation of uric acid from hypoxanthine and xanthine under normal physiological conditions. However, XD is nonfunctional in oxidising hypoxanthine and xanthine under hypoxic conditions. Therefore, XD is converted to its oxidised form, which is XO. The XO utilise O_2 as the electron acceptor, giving rise to $O_2^{\bullet-}$ and H_2O_2 (Ji & Leichtweis, 1997; Sjödin et al., 1990).

The concentration of XO and hypoxanthine can increase during hypoxia. Hence, when oxygen is restored (reperfusion), it can cause a burst of $O_2^{\bullet-}$ and H_2O_2 (Coombes et al., 2001; Goldfarb, 1999; Heunks et al., 1999)

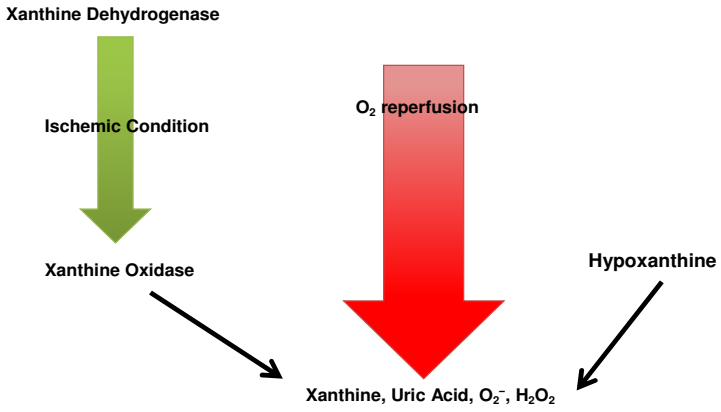


Figure 2.2 A suggested mechanism for the production of free radicals upon reoxygenation of ischemic or hypoxic tissues. Adapted from Gomes et al. (2012)

In 1987, Norman et al. found that hypoxanthine accumulated after intense muscular contraction because of adenine nucleotide degradation. Four years later, Sahlin et al. (1991) supported this finding; this author found that the concentrations of hypoxanthine and xanthine in the blood increased intensely in human subjects after intense exercise. Hellsten-Westing et al. (1993) discovered that the uric acid concentration increased in the plasma during arm muscle contraction implying that XO is activated. Radak et al. (1995) showed that, after repeated runs to exhaustion in high-intensity, plasma XO activity increased 10-fold and that plasma XO activity is associated with lactate concentration. This study proposed that XD was converted to XO via a Ca^{2+} activated protease and the source of the enzyme was from the endothelial cells of the muscle.

Allopurinol has been used to inhibit XO activity in previous study (Heunks et al. 1999; Viña et al. 2000). These studies provided additional evidence indicate that XO is one source of FR following exercise. Production of ROS by XO is not restricted to skeletal muscle and possibly would lead to oxidative stress for several hours following exercise. However, it seems unlikely that this mechanism is responsible for the intensified FR production during exercise because appearance of XO occur primarily after exercise (Vollaard et al., 2005).

The theory that XO plays an important role in FR production during exercise remains uncertain, even though hypoxanthine and xanthine tend to accumulate during intense muscle contraction. This feasibly occurs only during exercise when blood flow and oxygen supply to muscle are low such as ischemic exercise, or during exercise involves only a small muscle groups such as arm exercise (Ji & Leichtweis, 1997). Whereas during exercise which involves large muscle groups might not result in an observable accumulation of purine nucleotide degradation products as this type of exercise has sufficient oxygen supply to ensure the replenishment of ATP predominantly through mitochondrial oxidative phosphorylation (Sahlin et al., 1991).

However, in addition to the activity of XO during and after tissue ischemia, Gomez-Cabrera et al. (2010) provided more recent evidence to support the idea that XO is important in superoxide generation in the extracellular fluid after a non-damaging protocol of muscle contractions.

2.2.3 NADPH Oxidase Structure

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is another alternative mechanism of FR production during exercise (Bejma & Ji, 1999; Powers et al., 2011). Jackson (2008) hypothesised that NADPH oxidase contributes to the production of ROS during exercise in skeletal muscle. NADPH oxidase is typically inactive but, when it becomes activated, it can generate large amounts of $O_2^{\bullet -}$ that can be converted in H_2O_2 by the antioxidant superoxide dismutase during muscle contraction (Babior et al., 2002).

NADPH oxidase located in numerous cellular locations in muscle fibers such as the sarcoplasmic reticulum, transverse tubules, and sarcolemma; it generates superoxide during exercise by shifting electrons from NADPH to molecular oxygen (Halliwell, 2007; Powers & Jackson, 2008). This activity occurs in transverse tubules by depolarisation,

releasing superoxide to the cytosol of skeletal muscle cells (Espinosa et al., 2006; Hidalgo et al., 2006). NADPH oxidase influences calcium release by the sarcoplasmic reticulum through ryanodine receptor oxidation to generate $O_2^{\bullet-}$ (Xia et al., 2003).

Recently, studies investigated the production of intracellular ROS in skeletal muscle during muscular contraction from mitochondria and non-mitochondrial sources (Pearson et al., 2014; Sakellariou et al., 2013) and these studies concluded that NADPH oxidase activity is the main source of intracellular $O_2^{\bullet-}$ production during contractile activity and mitochondria play little role in this.

The sources of the extracellular ROS that are released from skeletal muscle remain uncertain. Theoretically, extracellular ROS originated from diffusion of intracellular ROS through cell membrane, thus these intracellular ROS may play a role in production of extracellular ROS. However, a recent notion from Jackson et al. (2016) proposed that the diffusion of H_2O_2 (produced by the conversion of $O_2^{\bullet-}$ activated by NADPH oxidase) from the inner side of muscle fibers to the extracellular space cannot occur because of the large H_2O_2 concentration gradient during muscular contraction between intracellular (100–200 nM) (Jackson, 2011) and extracellular (15–20 mM) (Vasilaki et al., 2006). This idea effectively excluded the theory that intracellular muscle sources play any role in contraction-induced oxidative stress outside the muscle fibers.

2.2.4 Neutrophils and the Inflammatory Response

Tissue damage may lead to an upsurge in production of reactive species from nonmuscle sources following an exercise bout. Polymorphoneuthrophils (PMN) are type of white blood cells that have a crucial role in protecting tissue from bacteria (Pyne, 1994). PMN migrate to areas of inflammation and release two primary factors for phagocytosis, which is lysozymes and $O_2^{\bullet-}$ during the acute phase response. Lysozymes enable the breakdown of damaged tissue, while $O_2^{\bullet-}$ is generated by

myeloperoxidase (MPO) and NADPH oxidase (**Figure 2.3**) (Pyne, 1994). Myeloperoxidase (MPO) exists in neutrophils and it is an iron-containing enzyme. It catalyses the transformation of hydrogen peroxide (H_2O_2) into hypochlorous acid (HOCl), an extremely potent oxidant (**Figure 2.3**) (Vollaard et al., 2005).

Previous studies reported that PMN levels (Quindry et al., 2003; Suzuki et al., 1996; Suzuki et al., 2003) and MPO levels (Bury & Pirnay, 1995; Camus et al., 1992; Pincemail et al., 1990; Suzuki et al., 2003; Wetzstein et al., 1998) remain elevated for hours. This inflammatory response is important to eliminate damaged proteins and infections; nevertheless these cells can release ROS and other oxidants that cause secondary damage, such as lipid peroxidation (Gomes et al., 2012).

Bøyum et al. (2002) demonstrated that respiratory burst activity of PMN and number of neutrophil increased following 65 minutes cycling at 75% $\dot{V}O_2$max. Ramel et al. (2004) also found that during short duration resistance exercise, which is less than 20 minutes, there was an increased in neutrophil number. This highlights the fact that even if oxygen consumption is only moderately increased during physical activity there is still an increment in plasma neutrophils. In support of this idea, Peake & Suzuki (2004) investigated the inflammatory response following a bout of exhaustive exercise; the authors found the levels of neutrophils intensified and correlated with the duration and intensity of exercise.

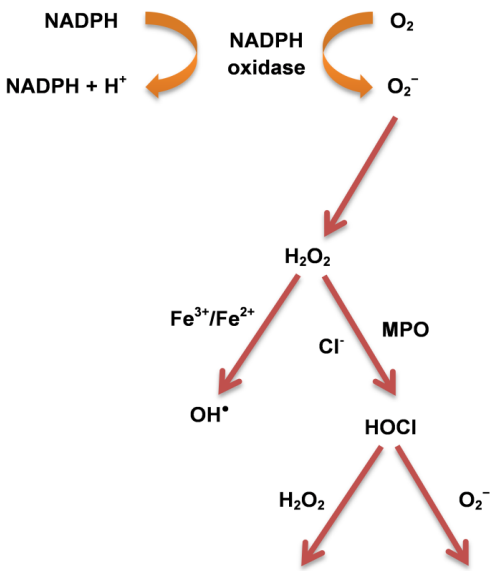


Figure 2.3 The production of reactive oxygen species by neutrophils. Adapted from Pyne (1994).

2.2.5 Autooxidation of Catecholamines

Under various stress conditions, for example heavy exercise, the heart releases noradrenaline from sympathetic nervous system. In the human body, the most abundant catecholamines are adrenaline, noradrenaline and dopamine. The level of circulating catecholamines would increase following exercise (Ghimire et al., 2012). Catecholamines activate β-adrenergic receptors to augment myocardial and skeletal muscle oxidative metabolism, thus potentially rising the ROS production via mitochondrial pathways (Ji & Leichtweis, 1997). The oxidation of catecholamines could yield the $O_2^{\bullet -}$, H_2O_2, and other non-oxygen derived species, which may reduce blood antioxidant concentrations such as glutathione, thus altering the redox (oxidation-reduction) balance (Halliwell, 2007; Powers & Jackson, 2008).

At rest, the muscles' concentration of ROS is low and only sufficient for force production. However, during muscle contractile activity there is an upsurge in ROS production. One point of view is that production of ROS is considered valuable for the muscle fibers' adaptation during both anaerobic and aerobic exercise (Gomez-Cabrera

et al. 2008; Gomez-Cabrera et al. 2009). Nonetheless, during strenuous exercise, the production of ROS could be greater than the antioxidant capacity of the muscles to scavenge the FR. This accumulation of ROS could oxidise proteins and lipids that might inhibit force production, thus influence the incidence of acute fatigue (Powers & Jackson, 2008; Reid, 2001). Furthermore, the high levels of ROS could also restrict the locomotor and bactericidal activity of neutrophils, cause oxidative damage to DNA, inhibit natural killer cells, damage cell membrane and other cellular compounds as well as result in a decline the proliferation of T lymphocytes and B lymphocytes (Niess & Simon, 2007; Sen & Roy, 2001).

2.2.6 Formation during Haemoglobin and Myoglobin Oxidation

Another theory has been proposed behind the mechanism of FR production during exercise which is involves haem proteins (Cooper et al., 2002) such as haemoglobin (Hb) and myoglobin (Mb). They contain iron, which enables the creation of primary ROS and enhances the reactivity of ROS generated by other pathways.

This alternative pathway involves haem protein auto-oxidation for example oxyhaemoglobin (oxyHb) and oxymyoglobin (oxyMb) (Vollaard et al., 2005). In the human body, approximately 3% of the total haemoglobin is transformed by auto-oxidation. This reaction increases during exercise would produces methaemoglobin and $O_2^{\bullet-}$ and releases superoxide radicals which are converted to hydrogen peroxide afterwards (**Equations 2 and 3**) (Brantley et al., 1993; Cooper et al., 2002; Gohil et al., 1988; Misra & Fridovich, 1972).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-} \text{ (**Equation 2**)}$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H+ \rightarrow H_2O_2 + O_2 \text{ (**Equation 3**)}$$

The reaction of methaemoglobin (metHb) or metmyoglobin (metMb) with hydrogen peroxide generate the production of two strong oxidants, ferric haem is oxidised to the

ferryl form (Fe^{4+} = $O_2^{\bullet-}$), with formation of protein bound FR (R•+) simultaneously (Gibson & Ingram, 1956) (**Equation 4**).

$$R\text{-}Fe^{3+} + H_2O_2 \rightarrow R^{\bullet+}\text{-}Fe^{4+} = O_2^{\bullet-} + H_2O \quad (\textbf{Equation 4})$$

Ferryl iron and free radicals that haemoglobin and/or myoglobin produce could react with a range of biological materials such as initiating the lipid peroxidation (Reeder & Wilson, 2001). Theoretically, both ($R^{\bullet+}$-Fe^{4+}= $O_2^{\bullet-}$) are considered to be harmful oxidants which reactly to the hydroxyl radical (Cooper et al., 2002). During ischaemia-reperfusion, myoglobin can be oxidised by auto-oxidation or by FR with the production of H_2O_2 (Brantley et al., 1993; Gunther et al., 1999). It can then interact with H_2O_2 and yield other radicals such as ferryl radicals or peroxyl radicals (Giulivi & Cadenas, 1998; Harel & Kanner, 1988; Kelman et al., 1994).

In summary, most available research reveals that ROS production increases concurrently with physical activity in almost all studied organs, tissues, and cells. There are many sites for producing ROS during or after exercise (**Figure 2.4**). Mitochondria could be the major site for ROS production during moderate intensity aerobic exercise. However, during or after exhaustive exercise, when energy depletion or ischemia-reperfusion takes place, XO activity might be a crucial source for ROS production. After exercise, macrophages, eosinophils, neutrophils and other cells in the immune system may also contribute to the ROS formation when tissue damage occurs**.**

Moreover, production of FR are likely to depend on the mode (aerobic, anaerobic), intensity (moderate, high), and duration (acute, chronic) of exercise because different types of exercise vary in their respective energy requirements, levels of oxygen consumption and mechanical stresses exerted on the organs, tissues and/or cells.

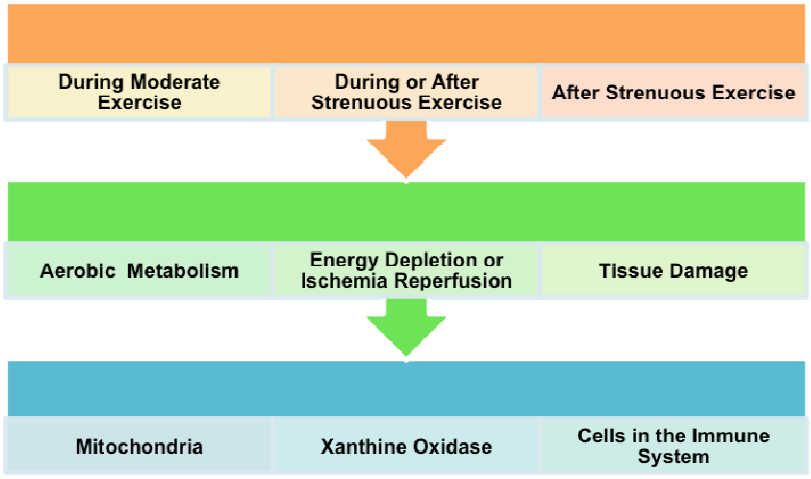


Figure 2.4 The potential sites and sources of reactive oxygen species (ROS). Adapted from Li (2013).

2.3 BIOLOGICAL EFFECTS OF FREE RADICALS

It is well defined that FR production and removal are constantly occurring at a basal level, consequently eliciting both positive and negative effects on physiological function. In living systems, this balance (FR production vs. antioxidant defence) assists in maintaining redox homeostasis (Allen & Tresini, 2000) in order to optimise cellular function.

Beyond this, exercise-induced FR might activate some redox-sensitive signaling pathways and induce the endogenous antioxidant defence system (Ji, 2002; Ji et al., 2006). In fact, both the positive and negative aspects of FR production in sport performance are currently considered (Fisher-Wellman & Bloomer, 2009; Nikolaidis et al., 2012; Yavari et al., 2015).

2.3.1 Positive Effects

Even though most studies have centered on the negative effects of FR, it has been commonly accepted that moderate levels of reactive oxygen species function as regulatory mediators in signaling processes and as initiators in regenerating "redox

homeostasis" (Reid et al., 1992; Rimbach et al., 1999; Sen, 2001; Sen & Packer, 1996). ROS appear to play such an important role in cellular signaling, particularly as they can serve as cell messengers (Reid et al., 1992; Sen & Packer, 1996; Rimbach et al. 1999; Murrant & Reid, 2001; Sen 2001). At low concentrations, ROS might help to maintain the muscle force production in non-fatigued muscle (Powers & Jackson, 2008). Reid et al. (1993) were the first described a theoretical model, which explained the relationship between muscle redox balance and isometric force production. This theory predicts that an optimal cellular redox state occurs when there is balance between the rates of ROS production with cellular antioxidant defences' capacity and this condition are considered ideal for muscle force production (Reid et al., 1993). Therefore, deviation from the optimal redox balance could lead to loss of force production. In addition, ROS are also known to be involved in facilitating glycogen repletion, in drug detoxification as well as in enzyme activation (Jenkins, 1988). Furthermore, release of $O_2^{\bullet-}$, H_2O_2, and NO● during the oxidative burst of phagocytosis and macrophages helps to clear out dead cell material, which speeds the repair process (Valko et al., 2006).

Studies have indicated that during exercise-induced oxidative stress, ROS could activate redox-sensitive signaling pathways such as nuclear factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) (Ji, 2007) both in humans (Vider et al. 2001) and in animals (Hollander et al., 2001). Activation of these pathways induces antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and inducible nitric oxide synthase (iNOS) (Ji, 2002; Ji et al., 2006; Morgan & Liu, 2011; Yavari et al., 2015). In other words, the production of FR during non-exhaustive moderate exercise especially may act as the best antioxidant.

2.3.2 Negative Effects

On one hand, the low level production of FR is essential for intracellular signaling,

immune function and normal cellular redox status. On the other hand, an extreme level of production can damage lipids, proteins and DNA as well as disrupt the function of cell leading to cell death. Because of their high reactivity, these FR are able to distort other molecules and consequently damage the structure of the cell and hinder cell function. For example, lipid peroxidation occurs when $O_2^{\bullet-}$, H_2O_2, and OH● attain the protons adjacent to double bonds in unsaturated fatty acids, such as in cell membranes; it begins a deformation reaction to these fatty acids forming lipid peroxides, resulting in poorly functioning cell membranes (Valko et al., 2004; Welles Kellogg & Fridovich, 1975). In the same way, some researchers found that OH●, NO●, and ONOO─ can cause DNA damage by oxidising the nucleotides that can lead to tumours (Valko et al. 2006). According to Stamler et al. (2008), nitric oxide has a capability to bind with the cysteine groups on proteins, called S-nitrosylation, modifying the protein's tertiary structure and function. In addition, ONOO─ also has the ability to denature proteins permanently in a similar manner, making them non-functional (Beckman & Koppenol, 1996). Kobzik et al. (1994) suggested that nitric oxide also has a direct inhibitory effect on muscle fiber contraction.

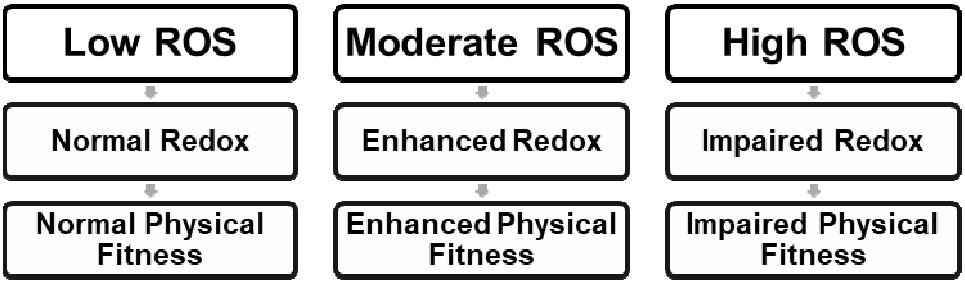


Figure 2.5 Potential effects of reactive oxygen species (ROS). Adapted from Li (2013)

Based on **Figure 2.5**, it can be concluded that it is necessary to maintain redox homeostasis with adequate levels of ROS. While optimal levels of ROS can enhance redox homeostasis and improves physical fitness. However, an extreme level of ROS may create a stressful signal that can disrupt the redox homeostasis.

In summary, FR is not essentially harmful however the redox-balance system could become imbalance due to chronic exposure or excessive production of FR, (free radicals > antioxidant defence). This condition could potentially shift redox homeostasis towards a more oxidising environment as a result inducing oxidative damage, inflammation, ill-health, and disease.

2.4 EXERCISE-INDUCED OXIDATIVE STRESS: EXPERIMENTAL EVIDENCE

Since the initial breakthrough of increased lipid peroxidation following acute aerobic exercise in 1978 by Dillard and colleagues, an abundance of studies has explored the effects of exercise-induced oxidative stress over the past 30 years. These numerous investigations note an increase, decrease or no changes in various oxidative stress biomarkers following both acute aerobic and anaerobic exercise.

2.4.1 Aerobic Exercise

Most of the studies involve protocols including submaximal or maximal effort aerobic exercise either on a treadmill or cycle ergometer, with investigations utilising a graded exercise test (GXT), hypoxic exercise or environmentally extreme exercise to induce oxidative stress (**Table 2.3**). Laboratory-based protocols have mostly involved short- to moderate-duration of exercise (≤2 hours), while some of laboratory protocols and field-based tests involved a much longer duration of exercise (>2 hours).

2.4.2 Anaerobic Exercise

Anaerobic exercise includes a large variety of sport activities such as sprints, jumps or resistance exercise. Although the term anaerobic means "without oxygen", these studies also demonstrate a raise in oxidative stress after supramaximal exercise such as sprints, jumps, sets of jumps, resistance exercise (eccentric or concentric), intermittent running, Wingate tests on an ergocycle or sets of 50m or 100m swimming (**Table 2.4**).

Table 2.3 Selected human studies on the effects of aerobic exercise on markers of oxidative stress

Study (year)	Activity	Subjects	Markers	Effect
Lovlin et al. (1987)	Cycling at 40%, 70% and 100% $\dot{V}O_2$max	6 UT	MDA (at 40% VO_2max)	↓
			MDA (at 70% VO_2max)	↔
			MDA (at 100% VO_2max)	↑
Kanaley & Ji (1991)	Treadmill running test at 60% $\dot{V}O_2$max for 90 minutes	12 T	MDA	↔
			GPx	↔
			GR	↔
			CAT	↔
Camus et al., (1994)	Uphill treadmill walking (35 min at 60% $\dot{V}O_2$max)	8 UT	GSH	↔
			GSSG	↔
Laaksonen et al. (1996)	Cycling, 40 min, at 60% $\dot{V}O_2$max	13 UT	TBARS	↑
			TGSH	↔
			GSSG	↑
Margaritis et al. (1997)	Triathlon (long distance ~468min)	18 VT	TBARS – GSSG	↔
Marzatico et al. (1997)	Running (half-marathon ~90min)	6 T	MDA	↑
			CD	↔
			SOD – GPX	↑
			CAT	↑
Vasankari et al. (1997)	Running (31km ~180min)	22 VT	Tocopherol – TRAP	↑
			CD	↑
			Retinol – CoQ10	↔
Ashton et al. (1998)	$\dot{V}O_2$max test (ergocycle)	12 T	TAC	↑
			FR (ESR) – MDA – LH	↑
Child et al. (1998)	Running (half-marathon ~87min)	17 T	MDA	↑
			CK	↑

			TEAC – UA	↑
Alessio et al. (1999)	Run to exhaustion (treadmill)	12 UT	TBARS	↔
			PC	↑
			LH	↑
			ORAC	↑
Liu et al. (1999)	Running (marathon running times varied from 193 to 352 min)	11 VT 10 UT	Oxidised LDL	↑
			TRAP – UA	↑
			Thiols	↓
			Tocopherol – vit C – vit A	↔
Hellsten et al. (2001)	Two exercises to exhaustion at 90% $\dot{V}O_2$max (cycling ~20min for exercise 1 and ~15min for exercise 2)	8 T	Allantoin	↑
			UA (muscle)	↑
			GSH – cysteine – UA (plasma)	↔
Inal et al. (2001)	Swimming (800m ~480min)	10 T	CAT – GPX	↑
			GSH	↓
Mastaloudis et al. (2001)	Running (50km ~410min)	11 T	Isoprostane	↑
			UA – tocopherol – vit C	↑
Miyazaki et al. (2001)	$\dot{V}O_2$max test (ergocycle)	9 UT	TBARS – neutrophil FR production	↑
			Protein carbonyls	
			SOD – GPX – CAT	↔
				↔
Vider et al. (2001)	$\dot{V}O_2$max test (treadmill)	19 T	TBARS – CD	↑
			TAC – GSH – CAT	↑
			GPX – SOD	↔

Dawson et al. (2002)	Running (21km ~87min)	15 T	MDA	↑
			CK – myoglobin	↑
Chevion et al. (2003)	Walking (50km carried 35kg back load ~600min march)	29 T	CK	↑
		16 T	Protein carbonyls	↓
	Walking (80km carried 35kg back load ~1200min march)		UA	↑
Palmer et al. (2003)	Ultra-marathon (80km ~600min)	28 T	LH – F2-isoprostane	↑
			Vit C	↑
Aguiló et al. (2005)	Cycling (171km ~270min)	8 T	GSSG	↑
			UA – tocopherol	↑
			GPX	↓
McAnulty et al., (2005)	Running on treadmill at 50% $\dot{V}O_2$max in two different conditions:- 1. Hot (35°C, 70% humidity) until body temperature reached 39.5°C 2. Neutral (25°C, 40% humidity)	6 T	LPO	↑
			F2-isoprostanes	↑
Steinberg et al. (2007)	$\dot{V}O_2$max test (ergocycle) then 5 min cool down.	15 UT	TBARS	↑
			RAA	↓
			GSH	↓
Goto et al. (2007)	30 min cycle ride at 25%, 50%, 75% $\dot{V}O_2$max	8 UT	F2-isoprostanes	↑ (only at 75% $\dot{V}O_2$max)
Serrano et al. (2010)	Four days road cycling competition.	6 VT	LPO	↑
			Erythrocyte GPX	↑
			Erythrocyte GR	↔
			GSSG/GSH ratio	↔

Berzosa et al. (2011)	Three cyclo ergometric tests:- 1. Progressive exercise test ($\dot{V}O_2$max) 2. Strenuous test until exhaustion 3. Submaximal exercise (70% of the expected maximum workload) for 30 min.	34 UT	TAS CAT GR GPX SOD	↑ ↑ ↑ ↑ ↑
Turner et al. (2011)	Ultraendurance Race (233km ~1800min)	9UT	DNA damage LPO PC Reduced GSH	↑ ↑ ↑ ↑
Nikolaidis et al., (2012)	Two groups:- 1. Non muscle-damaging group- 45 min running on a level treadmill (70%–75% of $\dot{V}O_2$max) 2. Muscle-damaging group- 45 min running downhill treadmill at –15% gradient (70%–75% of $\dot{V}O_2$max)	20 UT	F2-isoprostanes PC Erythrocyte GSH Erythrocyte GPX Erythrocyte SOD Erythrocyte CAT	↑ ↑ ↓ ↑ ↑ ↑
Quindry et al., 2013	Three exercise trials (One hour stationary cycle exercise at 60% Wmax) :- 1. Cold (7°C, 40% humidity) 2. Room temperature (20°C, 40% humidity) 3. Warm (33°C, 40% humidity).	12 RA	TEAC FRAP LH PC	↑ (Warm) ↑ (Warm) ↑ (Warm) ↔
Kabasakalis et al. (2014)	2000m continuous freestyle swimming at the fastest possible.	30 T	8-OHdG (DNA oxidative damage) MDA PC	↑ ↑ ↔

			GSH	↑
			UA	↑
Sureda et al. (2015)	Running on treadmill for 45min at 70-85% $\dot{V}O_2$max in two different conditions:- 1. Temperate environment (11.6±0.8 °C and 48.3±3.0% humidity) 2. Hot, humid environment (32.4±0.5 °C and 76.6±0.5% humidity).	9VT	MDA	↑ (hot)
			Urinary 8-OHdG	↑ (hot)
			CAT	↑ (hot)
			SOD	↔
			PC	↔
			PON1	↑ (hot)
Wadley et al. (2016)	1. Low Volume-High Intensity Interval Exercise (Ten set of 1 min stages, cycling at 90% $\dot{V}O_2$max) for 19 min 2. High Intensity (Cycling at 80% $\dot{V}O_2$max) for 20 min. 3. Moderate Intensity (Cycling 60% $\dot{V}O_2$max) for 27 min	10 UT	LH	↑
			TAC	↑
			PC	↑

CAT = catalase; CD = conjugated dienes; CK = creatine kinase; CoQ10 = coenzyme Q10; ESR = electron spin resonance; FR = free radical; GPX = glutathione peroxidase; GR = glutathione reductase; GSH = glutathione; GSSG = oxidised glutathione; LDL = low-density lipoprotein; LH = lipid hydroperoxide; LPO = lipid peroxide; MDA = malondialdehyde; RAA = reduced ascorbic acid; SOD = superoxide dismutase; PON1 = paraxonase1; PC = protein carbonyl; T = trained; TAC = total antioxidant capacity; TAS = total antioxidant status; TBARS = thiobarbituric reactive substances; TEAC = trolox equivalent antioxidant capacity; TRAP = total radical antioxidant potential; ORAC = oxygen radical absorbance capacity; UA = uric acid; vit = vitamin; RA = recreational athlete; UT = untrained; VT = very trained; VO_2max = maximum oxygen consumption; ↓ indicates decrease; ↑ indicates increase; ↔ indicates no change(stable).

Table 2.4 Selected human studies on the effects of anaerobic exercise on markers of oxidative stress

Study (year)	Activity	Subjects	Markers	Effect
Sahlin et al. (1992)	Isometric knee extension at 60% 1RM intermittent – 80 min	7 UT	MDA GSH (blood) GSH (muscle) GSSG (blood and muscle)	↔ ↑ ↔ ↔
Saxton et al. (1994)	Elbow flexion – 70 max eccentric or concentric Actions	14 NRT	TBARS – CD – MDA Protein carbonyls	↔ ↑
Marzatico et al. (1997)	6 × 150m sprints	6 T	MDA – CD SOD – GPX CAT	↑ ↑ ↔
Ortenblad et al. (1997)	6 bouts of jumping – 30 sec each bout	8 JT 8 UT	MDA	↔
McBride et al. (1998)	Resistance training programme (8 exercises, 3 sets of each failure)	12 T	MDA	↑
Alessio et al. (1999)	Maximum hand grip dynamometer	12 UT	TBARS PC LH ORAC	↔ ↑ ↑ ↑
Alessio et al. (2000)	Isometric handgrip exercise at 50% MVC intermittently for ~15 min	12 T	PC MDA LOOH	↔ ↔ ↑

Childs et al. (2001)	Eccentric arm flexion (cybex) 3 × 10 reps at 80% RM	14 UT	LH – isoprostane CK – LDH – myoglobin SOD GPX	↑ ↑ ↑ ↔
Inal et al. (2001)	100m swim	9 T	CAT – GPX GSH	↑ ↓
Groussard et al. (2003)	Cycling – Wingate tests (30 sec)	8 T	ESR signals TBARS SOD – GSH GPX	↑ ↓ ↓ ↔
Groussard et al. (2003)	Cycling – Wingate tests (30 sec)	7 T	UA – vit C Tocopherol – vit A	↑ ↓
Lee & Clarkson (2003)	50 max eccentric actions with elbow flexors	60 NRT	MDA XO TGSH	↔ ↑ ↑
Ramel et al. (2004)	Resistance programme (10 exercises – max of reps at 75% 1RM)	7 T 10 UT	MDA CD (trained group) CD (untrained group) Vit A – tocopherol	↔ ↔ ↑ ↑
Goldfarb et al. (2005)	Eccentric resistance exercise	18 UT	PC – MDA GSSG GSH	↑ ↑ ↓

(Nikolaidis et al., 2007)	Two isokinetic exercise session separated by 3 weeks consisting of 75 lengthening knee flexions.	12 UT	GSH GSSG TBARS PC CAT UA	↓ (first session ↑ (first session) ↑ (first session) ↑ (first session) ↑ (first session) ↑
Demenice et al., (2010)	High intensity interval training of eight bouts of 100m maximum swims with 10min rest in between.	10 VT	TBARS GSH Ascorbic acid α-tocopherol AOPP	↑ ↑ ↑ ↔ ↔
Kabasakalis et al. (2014)	Set of six 50-m maximal freestyle swimming bouts	30 T	8-OHdG (DNA oxidative damage) MDA PC GSH UA	↑ ↑ ↔ ↑ ↑

CAT = catalase; CD = conjugated dienes; CK = creatine kinase; ESR = electron spin resonance; GPX = glutathione peroxidase; GSH = glutathione; GSSG = oxidised glutathione; JT = jump trained; LDH = lactate dehydrogenase; LH = lipid hydroperoxide; LOOH = lipid hydroperoxides; max = maximum; MDA = malondialdehyde; NRT = non-resistance trained; PC = protein carbonyl; AOPP = advanced oxidation protein products; reps = repetitions; RM = repetition maximum; SOD = superoxide dismutase; T = trained; TBARS = thiobarbituric reactive substances; TGSH = total glutathione; UA = uric acid; UT = untrained; vit = vitamin; XO = xanthine oxidase; ↓ indicates decrease; ↑ indicates increase; ↔ indicates no change (stable).

From work over the past thirty years, Fisher-Wellman & Bloomer (2009) clearly stated that exercise with sufficient intensity, volume and duration can increase free radical production, which may initiate the oxidation of numerous biological molecules (lipids, proteins, DNA). Based on **Table 2.3** and Table **2.4**, it could be speculated that both type of exercise, aerobic and anaerobic may induce the oxidative stress. According to Vollaard et al. (2005), oxidative stress is a required consequence of exercise that takes place when the exercise intensity is sufficiently high despite the training status.

It is remarkably well documented that exercise-induced oxidative stress is related to destruction of bodily molecules in a variety of tissues, such as lipids, protein and DNA (Alessio, 1993). In addition to cellular destruction, extreme levels of FR have been shown to contribute of muscle fatigue, have a harmful effect on skeletal muscle contraction and negative impact on performance (Barclay & Hansel, 1991; Novelli et al., 1990; Shindoh et al., 1990). Furthermore, antioxidant deficiency that leads to oxidative stress has been claimed to impair endurance performance during exhaustive exercise (Coombes et al., 2002; Davies et al., 1982; Gohil et al., 1986).

2.5 HYPERTHERMIA, OXIDATIVE STRESS AND EXERCISE

Human body temperature homeostasis is maintained through a complex series of regulatory physiological mechanisms; preserving equilibrium between the heat production of the body and the heat lost or dissipated to the environment. Exercise and/or body exposure to a warmer or more humid environment could disturb this heat equilibrium due to the impairment of heat dissipation mechanisms, leading to the accumulation of metabolic heat and hyperthermia (elevated core body temperature) (González-Alonso et al., 2000).

It has been postulated that, there is a possible linkage between hyperthermia and oxidative stress. Cells exposed to heat stress induced ROS production such as superoxide anion ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) (Belhadj Slimen et al., 2014; Davidson & Schiestl, 2001; Flanagan et al., 1998; Katschinski et al., 2000). Thus, heat stress could be a supplementary factor that induces oxidative damage to DNA, proteins and lipids (Bruskov et al., 2002; Grasso et al., 2003; Zhao et al., 2006).

Increases in environmental heat stress during exercise might lead to increase in core body temperature known as hyperthermia. Several studies have examined whether heat stress increases oxidative stress in humans during exercise (Laitano et al., 2010; Morton et al., 2007; Ohtsuka et al., 1994). A substantial increase of F2-isoprostanes was observed in men whose core body temperature increased to 39.5°C during exercise at 50% $\dot{V}O_2$max in a hot environment compared to the men who performed the same intensity of exercise at a thermoneutral environment (Laitano et al., 2010). However, at the end of this protocol, participants were dehydrated by approximately 3% of their initial body mass. It is it is therefore difficult to conclude that oxidative stress was exclusively induced by heat stress during exercise, as the responses observed in this study were, at least in part, related to dehydration (Hillman et al., 2011, 2013; Paik et al., 2009).

Other studies have demonstrated that elevated core (38.9ºC) and muscle temperature (39.5ºC) did not induce oxidative stress (Morton et al. (2007)), although participants in this study did not perform exercise. This study was attempting to mimic the thermal stress of exercise by immersing the leg in a warm water tank with temperature maintained at approximately 45ºC.

Interestingly, Laitano et al. (2010) discovered that heat stress, independent of exercise, increased oxidative stress. This study also found that combined heat stress with exercise raised glutathione (GSH) and glutathione disulfide (GSSG) level at the same time decrease superoxide dismutase (SOD) activity but did not affect the plasma isoprostane concentrations. Cycling at moderate-intensity (60% of Wmax) followed by recovery for 3 hours in a warm environment (33°C, 40% humidity) elicited oxidative stress response compared to cycle at cold (7°C, 40% humidity) and room (20°C, 40% humidity) environment.

Similarly, Sureda et al., (2015) reported that exercise for 45min at 75-80% of VO_2max in hot and humid environment (32ºC and 77% humidity) increased selected oxidative stress biomarkers when compared to temperate environment (12ºC and 48% humidity). These studies controlled the level of dehydration among participants, therefore, the results suggest the combined impact of both heat and exercise on examining exercise induced oxidative stress.

This inconsistency of results could be due to many factors such as the heat stress level (temperature), the extent of the resultant hyperthermia, dehydration status (control or did not control), experimental model (exercise or without exercise), exercise intensity (low, medium or high), exercise duration (short or long) and exercise type (aerobic or anaerobic) as well as different analysis of oxidative stress biomarkers. Despite the results inconsistency, evidences suggested that environmental temperature could play a crucial role to consider when evaluating exercise-induced oxidative stress and performance among athletes.

Stressors such as heat and exercise could trigger the first defence mechanism against

stress, which involve the following molecules: uric acid, glutathione, α-tocopherol, ascorbic acid, Q coenzyme, glutathione peroxidase, superoxide dismutase and many other compounds. It could also involve the second defence mechanisms, which are heat-shock proteins (HSPs) as a response to stress. Due to the increased HSPs levels induced by oxidative stress, these proteins are also reported to have an antioxidant effect (Fehrenbach & Northoff, 2001). The next section will briefly describe the role and response of heat shock proteins.

2.5.1 Heat Shock Proteins

During exercise, contracting muscles produced a significant amount heat and then the heat transferred from the contracting muscles to the skin surrounding the exercising limbs and body core via the circulating blood (González-Alonso et al., 2000). Synthesis of heat shock proteins can be induced by various stimuli such as stress agents (e.g. hyperthermia, hypothermia, oxidative stress, ultraviolet radiation, hypoxia, amino acid changes and heavy metals) and alterations in physiology state (e.g. cell cycle, growth factors and viral infection) (Kalmar & Greensmith, 2009; Kregel, 2002).

Heat shock proteins (HSPs) are the most highly conserved stress response proteins during evolutionary history (Kültz, 2005). HSPs exist in numerous subfamilies distinguished based on their molecular weight (kDa) and can be divided into five subfamilies which is HSP100, HSP90, HSP70, HSP60 and HSP27 (Kalmar & Greensmith, 2009; Kregel, 2002). HSPs are considered as proteins, which are universally expressed during stress such as exercise and exposure to extreme environmental temperatures.

Intracellular HSPs are the family of stress response protein which is involve with a multiple cytoprotective function including molecular chaperones which is necessary in inhibiting the

aggregation of folded protein, assisting the correct protein refolding and transferring the protein safely to the correct compartment (Lancaster & Febbraio 2007; Morton et al. 2006; Ghazanfarp & Talebi 2013).

In addition, HSPs also play a role outside the cells (extracellular). Under stress conditions, they could be released from cells into the extracellular environment or enter the systemic circulation and may interact with a wide range of target cells (Calderwood et al., 2007). Extracellular HSPs (eHSPs) have been suggested as a form of cellular messenger in response to the stress, injury, infection and cell damage. They have been demonstrated to activate innate immune response by binding to receptors on the damaged cell surface to protect from subsequent insults (Borges et al., 2012; De Maio, 2011; Jolesch et al., 2012).

Intracellular HSP synthesis is initiated by the activation of 'heat shock transcription factors' (HSFs) by a signaling cascade. HSFs exist as inactive monomers in the cytosol, attached to HSPs and form HSP-HSF complexes (Gupta et al., 2013). Upon activation in response to stress, they are separated from HSP and phosphorylated by protein kinases and then formed trimer resulting binding to DNA in the cytosol. HSF trimer complexes enter the nucleus where they bind with specific sites on HSP gene promoter regions known as 'heat shock elements' (HSEs) (Sreedhar et al., 2000). HSP mRNA is transcribed then leaves the nucleus, enter cytosol where the new HSP is synthesized (**Figure 2.6)**.

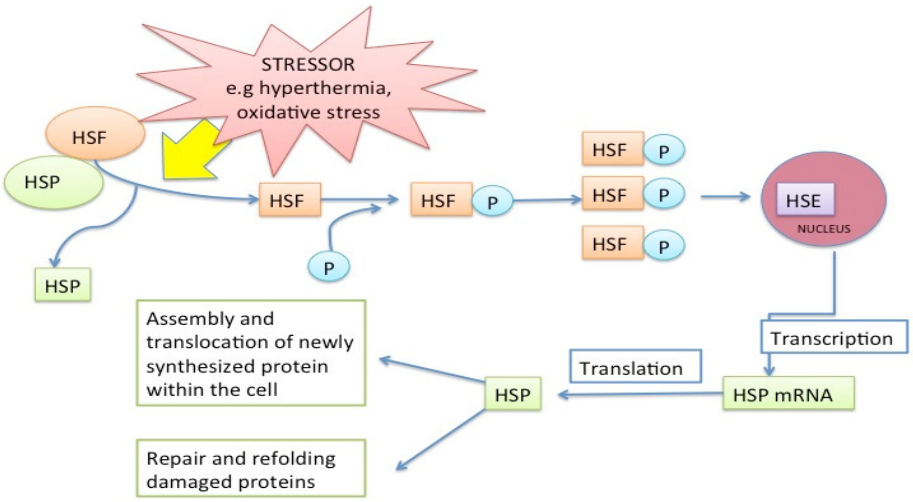


Figure 2.6 A schematic diagram of the mechanism of HSPs synthesis. Denatured proteins accumulate in response to physiological stressors, and activate HSP-HSF complexes. HSFs are phosphorylated by protein kinase and formed trimer in cytosol then enter nucleus to bind with the HSEs. HSP mRNA is then transcribed and leaves the nucleus. New HSPs are synthesized and ensure the repair of denatured proteins. Adapted from Belhadj Slimen et al. (2014)

Extracellular HSPs (circulating) were first reported in 1980s by the researches of Tytell et al. (1986) and Hightower & Guidon (1989). These first discoveries were disregarded since they were against the conventional theory that HSPs are only released in the intracellular environment and can only be detected in the extracellular environment after necrosis. After more than 10 years, the interest in HSPs was reborn since the study by Basu et al. (2000) revealed that the presence of eHsp70 are released from cell necrosis could act as a modulator of the immune system. Hunter-Lavin et al. (2004) demonstrated that the release of Hsp70 was not due to cell necrosis, confirming the earlier observations of Hightower & Guidon's (1989). Therefore, extracellular HSPs could probably be released by both passive (necrosis) and active (secretion) mechanisms.

There are several possible mechanisms for eHSP release and these have been reviewed elsewhere (De Maio & Vazquez, 2015; De Maio, 2011, 2014). Evidence suggests that the exosomal pathway is among the most important pathway for releasing eHSPs (Clayton et al., 2005; Gastpar et al., 2005; Lancaster & Febbraio, 2005b; Tytell, 2005). In addition, an endolysosomal-dependent pathway has been proposed (Mambula & Calderwood, 2006). In this process, Hsp70 is translocated into lysosomes via an ATP-binding cassette (ABC) transporter, where the protein is secured from degradation and transported to the extracellular space via the endocytic process. Nylandsted et al. (2004) supported these findings by demonstrating the presence of Hsp70 in the lumen of lysosomes. Moreover, another mechanism that has been proposed is that (Vega et al. (2008) Hsp70 is embedded within the plasma membrane before release into the extracellular environment and this membrane-bound Hsp70 is capable of activating macrophages serving as a danger signal.

A recent study investigated the response between eHsp72 and iHsp72 to exercise heat stress and recovery over 24 hours (Lee et al., 2017). This study discovered that eHsp72 concentrations corresponded to periods of exercise heat stress and recovery, where 15% post-exercise increases were observed to return to baseline during recovery. In contrast, the response of iHsp72 to exercise heat stress remained elevated and high even after 24 hours recovery (~2.5-fold baseline values). These data suggest that iHsp72 might be a better marker of ongoing effects of stress over 24 hours and eHsp72 could be an effective marker of a single exercise bout and accurately represents whole body stress and recovery periods.

HSPs are intrinsically linked with oxidative stress (Dimauro et al., 2016; Fittipaldi et al., 2014; Kalmar & Greensmith, 2009) and, thus, we decided to analyse HSP concentrations

in the current study. Heat stress also was suggested to be one of the environmental factor that play a role in stimulating ROS production because of similar responses observed following heat stress compared with exposure to oxidative stress (Belhadj Slimen et al., 2014; Périard et al., 2012). Although there are numerous subfamilies of HSP, the particular interest for this thesis are HSP70 (70 kDa) and HSP90 (90 kDa) families, thus only these will be discussed with regard to their induction and function during exercise.

2.5.1.1 HSP70 and HSC70

The 70 kDa family of HSPs is one of the HSPs that have been studied extensively (Morton et al., 2006). Proteins in the HSP70 family are produced in response to different stimuli even though they share common protein sequences. For example, the Hsp72 or Hsp70 is highly inducible and its production is increased in response to multiple stressors such as hyperthermia, exercise, hypoxia and ROS (Yamada et al. 2008), whereas the Hsp73 or Hsc70 is constitutively produced.

The evidence reviewed seems to suggest that physical exercise increases Hsp72 in various tissue in several mammalian species (Locke & Noble, 1995; Naughton et al., 2006; Sadowska-Krępa et al., 2006; Yamada et al., 2008). Both Hsp72 gene (Febbraio et al., 2002; Febbraio & Koukoulas, 2000; Puntschart et al., 1996; Walsh et al., 2001) and/or protein (Khassaf et al. 2001; Thompson et al. 2001; Morton et al. 2006) expression are increased in human skeletal muscle induced by exercise.

The role of temperature as an inducer for the cellular stress response was supported by the attenuation of Hsp72 expression when exercising in the cold environment (Hamilton et al., 2001). The expression of Hsp70 and Hsc70 in vastus lateralis muscle was elevated following an exercise that resulted in increases in core and muscle temperature by 1.7°C

and 3.8°C respectively (Morton et al. 2006). This may indicate that exercise-associated hyperthermia is involved in the exercise-induced production of HSPs in humans (Febbraio & Koukoulas, 2000; Lancaster & Febbraio, 2005a).

Whereas existing research demonstrated previously that circulating levels of Hsp72 are induced in response to several disease states, human study by (Walsh et al., 2001) was the first to demonstrate exercise induced the release of eHsp72 in the systemic circulation. However, this finding was not followed by elevation of Hsp72 in contracting muscle (intracellular), suggesting that contracting skeletal muscle is not the tissue source of the exercise-induced increase in the eHsp72 concentration, eHsp72 might be released from other tissues or organs.

Even though Hsp72 gene and protein expression are upregulated in human contracting skeletal muscle, they are not released to the extracellular compartment as measured by arterio-venous difference (Febbraio et al., 2002). It may indicated that eHsp72 is released from lysed muscle cells and intact muscle cells are not responsible in releasing eHsp72 into the circulation, but this stressed muscle cells synthesised Hsp72 to play a part in intracellular protection. However, the theory whether eHsp72 is released form damaged cells is yet to be determined. Subsequent study by the same author revealed that eHsp72 concentrations in blood circulation are release from human hepatosplanchnic tissue (Febbraio et al., 2002).

Other human studies have demonstrated that human brain possess the ability to release Hsp72 when induced by exercise (Lancaster et al., 2004) and leukocytes could be another source of Hsp72 releasing, as they have been found actively secrete Hsp72 (Hunter-Lavin et al., 2004). Hormones are also believed to be involved in stimulating eHsp70 during

exercise. It has been suggested that adrenaline in humans (Martin et al., 2006) and noradrenaline in animals (Johnson et al., 2005) play a role in stimulation of eHsp70. In animal studies, it also have been discovered that exercise induced significant increases in Hsp72 in the liver (Kregel & Moseley, 1996; Salo et al., 1991).

While Hsp70 is known as a stress inducible heat shock protein, Hsc70 is a constitutively expressed molecular chaperone in non-stressed cells, which belongs to HSP70 family. One of the Hsc70 functions is to uphold the protein homeostasis in both normal and stressed conditions (Liu et al., 2012). Hsc70 with newly synthesized Hsp70 could form a stable complex in response to heat shock (Brown et al., 1993). Exercise has been observed to induce the expression of Hsc70 in human skeletal muscle (Morton et al. 2006). The pattern of expression in muscle Hsc70, reflects the expression of Hsp70 and Hsc70, which increases significantly from pre-exercise levels to peak levels, typically occurring 48-72 hours post-exercise (Morton et al. 2006). In response to environmental heat stress, animal studies have found that both Hsp70 and Hsc70 are elevated in fish living in elevated water temperature (34.4±0.6 °C) compared with normal water temperatures (25.4±4.7°C) (Oksala et al., 2014).

Interestingly, a previous study also identified that Hsc70 is required for the HSF-1 activation and regulation during heat stress and subsequent target gene expression in mammalian cells (Ahn et al., 2005). This study demonstrated that carboxyl-terminal region of HSF-1 interacts directly with the substrate-binding domain of Hsc70 and form Hsc70–HSF-1 complex. Upon heat shock, this complex is translocated into the nucleus. In response to heat shock, Hsc70 is required for both the trimerisation of HSF-1 and HSF-1 mediated gene expression as well as induces the HSF-1 DNA-binding activity. Knocking down the expression of Hsc70 greatly reduced HSF-1 activities, thus this study indicating

that Hsc70 plays as a critical role of HSF-1 mediated cell survival in response to cellular damage.

2.5.1.2 HSF-1

The expression of HSPs is under the control of family of heat shock factors (HSFs) (Morimoto, 1998; Morimoto et al., 1996). HSF represents a family of transcription factors induced by both stressful and non-stressful stimuli. There are four isoforms of mammalian HSF have been discovered; HSF-1, 2, 3 and 4 (Åkerfelt et al., 2010; Morimoto, 1998; Noble & Shen, 2012) however HSF-1, 2 and 4 are ubiquitously present in humans and HSF-3 present in chickens (Morimoto, 1998). HSF-1 is expressed in heart, ovary, brain and placenta (Sarge et al., 1991) while, HSF-2 in postnatal tissue (Rallu et al., 1997) and HSF-4 in brain and lung (Tanabe et al., 1999).

As described above (**Figure 2.6**), several stressors (oxidative stress, heat shock, ATP depletion, hypoxia, exercise and pH alteration) could lead to the production of oxidised or misfolded protein, which induce the dissociation of HSF-1 from HSPs. HSF-1 exists as a monomer in the cytoplasm and the nucleus under basal conditions (Locke, 1997), possibly bound to HSP. Upon stress, this would change HSF-1 from its inactive monomer to trimer form (trimerisation), followed by hyperphosporylation and translocation to the nucleus, then binding with the heat shock element (HSE) of HSP genes and subsequent transcription of HSPs (Wu, 1995; Zuo, Baler, Dahl, & Voelimy, 1994).

Heat shock response rapidly activates various signaling pathways. There are four major signaling pathways that have been suggested (Nadeau & Landry, 2007). It consisted of three mitogen-activated protein kinase (MAPK) pathways, namely the extracellular signal regulated kinase (ERK), c-jun terminal kinase (JNK) and p38 as well as protein kinase B

(PKB) pathway which needs the prior activation of phosphatidylinositol 3-kinase (PI3K) (Nadeau & Landry, 2007).

Disruption in cellular redox homeostasis in response to thermal or oxidative stress is a common feature of stress-induced activation of HSF-1 in mammalian cells (McDuffee et al., 1997). Therefore, exercise induced oxidative damage to proteins which subsequently activate and modified HSF-1 by free radicals might happen (McArdle et al., 2001; Noble & Shen, 2012). Paroo et al. (2002) identified that protein denaturation induced by intense treadmill exercise probably involved in the induction of HSF-1-HSE binding activity in rat myocardium.

Palomero et al. (2008) was the first attempt to investigate the elevation of DNA binding of HSF in human muscles following an intermittent exercise protocol even though this study failed to prove that the increase of DNA binding of HSF when both muscle and core temperature was significantly increased as a consequence of performing the exercise protocol under ambient heat stress (40°C). However, in animal studies, the up-regulation, activation, translocation and binding of HSF-1 have been reported in cardiac induced by chronic exercise or after single acute bout of exercise (Melling et al., 2004; Sakamoto et al., 2006).

The data collected so far, for human study regarding exercise-induced oxidative stress in activation of HSF-1 are limited. Regardless the limited human study regarding effect of exercise on HSF-1 expression, the role of exercise-induced ROS in controlling influence of heat shock protein response has been reviewed elsewhere (Dimauro et al., 2016; Fittipaldi et al., 2014).

2.5.1.3 HSP90

Hsp90 represents one of the most abundant proteins in mammalian cells (Jakob, 1996; Sidera & Patsavoudi, 2008; Tsutsumi & Neckers, 2007), present in both the cytoplasm (Haverinen et al., 2001) and nucleus (Picard, 2006). It acts as a chaperone protein which functions to prevent damaged proteins from aggregation, unfold aggregated proteins, and refold damaged proteins by maintaining them in a folding competent state (Taipale et al., 2012; Taipale et al., 2010; Young et al., 2001).

Furthermore, Hsp90 involved in hormone signaling pathway, the glucocorticoid receptor (GR) (Picard et al., 1990) and steroid hormone receptor (SHR) (Pratt & Toft, 1997) are the most thoroughly studied examples of a hormone receptor whose function is crucially dependent on interactions with Hsp90 for proper functioning.

Study also indicated that Hsp90 interacts with the 26S proteasome and plays a principal role in the assembly and maintenance of the 26S proteasome which 26S proteasome is an integral part of the cell's mechanism to degrade proteins (Imai et al., 2003), indirectly Hsp90 involved in protein degradation.

Hsp90 have been associated with stress and exercise. Locke et al. (1990) reported that treadmill exercise induced the increase of various types of HSPs in spleen cells, lymphocytes and skeletal muscle cells in rats. One of the HSPs increased has a molecular mass of 90 kDa and it is probably that this protein was Hsp90.

However, Fehrenbach et al. (2000) exposed that the expression of Hsp90 in leucocytes remained unchanged after half marathon and that Hsp90 levels were not influenced by the participants training status. Similar to Shastry et al. (2002) demonstrated that Hsp90 levels

in leucocytes are not significantly affected by exercise and the levels were similar between trained and untrained participants.

Similar to Hsp70, stress-induced synthesis of Hsp90 may interact with HSF-1 which form HSf-1-hsp90 complex (Anckar & Sistonen, 2011; Hentze et al., 2016; Zou et al., 1998). During stress exposure, Hsp90 dissociate from the complex and bind to unfolded/misfolded protein. Upon dissociation from Hsp90, HSF-1 undergoes trimerisation, phosphorylation and translocate to the nucleus. Therefore, Hsp90 acts as an HSF-1 regulator to regulate the cellular stress response (Guo et al., 2001; Nadeau et al., 1993). Multi-component chaperone complex associating several HSPs (e.g Hsp90 and Hsp70) are crucial for optimal protection, thus signifying that inhibition of Hsp90 may delays and impairs heat stress recovery (Duncan, 2005).

2.6 ANTIOXIDANT DEFENCE SYSTEM

Due to the fact that exercise is one of the primary source of ROS generation and production of free radicals in the working muscle that may induce in lipid, protein and DNA damage, it is not surprising that human body also have a complex network of antioxidant defence mechanism that acts as a defence system counterbalancing the free radicals and reactive species. This process allows for the maintenance of redox balance.

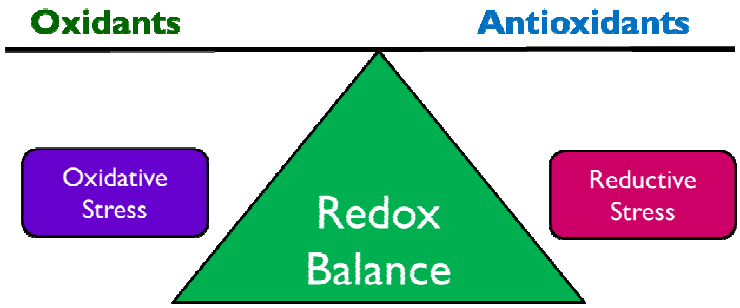


Figure 2.7. Illustration of the relationship between oxidants and antioxidants in the determination of cellular redox balance. Adapted from Powers et al. (2004).

Based on **Figure 2.7**, increase in either oxidants or antioxidants may disrupt the cellular redox balance. Oxidative stress occurs when oxidants surpass the available content of antioxidants. On the contrary, reductive stress happens when antioxidants are more numerous than the oxidants exist in the cell.

Abundant of antioxidants exists in the cell. These antioxidants can be divided into endogenous (synthesised in the body) and exogenous (absorb through diet) antioxidant. Both types of antioxidant interact to each other in order to form a cooperative network of cellular antioxidant. Both types apply several approaches to protect against reactive oxygen species induced damage. These include modify the active ROS into less active molecules and inhibit the conversion of least ROS into more damaging forms, for example conversion of hydrogen peroxide to the hydroxyl radical.

Endogenous antioxidant presents in two types, which is enzymatic and non-enzymatic. Primary enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Gomes et al., 2012; Noori, 2012; Powers et al., 2004). Each of these enzymes is

responsible for different ROS and they are located in different cellular compartments (**Table 2.5**). Other than primary enzymatic antioxidants, human body also been equipped by secondary enzymatic antioxidants such as glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione-S transferase and ubiquinone which is work directly to neutralise ROS by decreasing the peroxides level and continuously supplying the NADPH and glutathione for primary antioxidant enzymes to maintain their proper functioning (Noori, 2012).

Table 2.5 Primary enzymatic antioxidants.

Enzymatic antioxidants	**Location**	**Properties**
Superoxide dismutase	Both mitochondria and cytosol	• In skeletal muscle cells, 65-85% found in cytosol, remaining 15-35% in mitochondria • Catalyses reaction of superoxide radicals into oxygen and H_2O_2.
Glutathione peroxidase	Both mitochondria and cytosol	• Present in muscle cells with the greatest activity in slow twitch muscle fibres (type I) which have higher oxidative capacity. • Removal of a wide range of hydroperoxides and H_2O_2.
Catalase	Cytosol and in mitochondria of heart	• Can be found in higher concentration in type I muscle fibres • Primary function is to degrade H_2O_2 into H_2O and O_2 • Has lower affinity to H_2O_2 compared with GPX.

Non-enzymatic antioxidants include glutathione (GSH), vitamin C, vitamin E, beta carotene, uric acid, lipoic acid, bilirubin and many more. Similarly to the enzymatic antioxidants, non-enzymatic antioxidants exist in different cellular compartments and

generate distinct antioxidant properties, which maximise their effectiveness. Some non-enzymatic antioxidant can be exogenous antioxidant which is can be absorbed through diet such as Vitamin C, Vitamin E, beta-carotene and flavonoids (Birben et al., 2012; Gomes et al., 2012; Noori, 2012).

Regular exercise is prescribed as one's resistance to FR damage (Gomez-Cabrera et al., 2008). Regular exercise with low (40 to 60% maximal heart rate; HRmax) to moderate (of 60-75% HRmax) intensity could improve cellular antioxidant defence by enhancing the antioxidant enzyme activity such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Aldred & Rohalu, 2011; Elosua et al., 2003) and protecting the body by modifying the immune responses. However, high exercise intensity (75-90% HRmax) may deplete the pool of antioxidant defence and who exercise strenuously and rigorously only on one occasion could be the most at risk for oxidative damage to cells (Gomez-Cabrera et al., 2008; Lamina et al., 2013; Radak et al., 2014; Turner et al., 2011; Valko et al., 2007).

2.6.1 Antioxidant Supplementation

Antioxidant supplementation has become more common among both professional athletes and amateur sportspersons. Review paper from Peternelj & Coombes (2011) revealed that since early 1970's even until now, there are more than 150 studies investigating the effects of antioxidant supplementation to reduce oxidative stress and muscle damage induced by exercise, promote recovery after exercise and enhance exercise performance.

Existing research has demonstrated that the production of FR is high after intense exercise, may disrupt the redox balance and impair the immune response (Aguiló et al., 2005; Nieman, 1994; Sureda et al., 2005). During exercise, the degree of oxidative

damage not only influenced by the level of free radical production, but also the ability of antioxidants' defence system. This is despite the fact that the body has its own intricate antioxidant defence system, which depends on dietary intake of vitamins, minerals as well as the endogenous production of antioxidant compounds such as glutathione, uric acid and coenzyme Q (Valko et al., 2007).

Antioxidant supplementation may aid in protecting from cellular oxidative damage by maintaining the redox balance and assisting in recovery by boosting the immune function after intense exercise, thus improve athletic performance (Close et al., 2016; Tauler et al., 2002; Tauler et al., 2003, 2008). Antioxidants may protect cells from FR damage in several ways; bind to free radicals, inactivate or kill them and augment the body's defense system (Speakman & Selman, 2011). For example, antioxidants protect lipids from lipid peroxidation by offer their own electrons to FR. When FR gains the electron from an antioxidant it may prevent the FR to attack the cell, as a result the chain reaction of oxidation is destroyed (Dekkers et al., 1996). Considering exogenous antioxidant supplementation to improve performance, recent finding (Vida et al., 2017) suggested that athletes should taking additional exogenous antioxidants with caution.

It is debatable whether the body's natural antioxidant defense system is sufficient to counterbalance the increase in FR with exercise or whether additional supplements are required. Interestingly, literature has emerged controversial issue about FR act as signalling molecules to stimulate antioxidant enzyme synthesis during exercise that leads to favourable exercise induced adaptations (Ji et al., 2006; Radak et al., 2014) but antioxidant supplementation could hamper this adaptations (Gomez-Cabrera et al., 2009). This adaptation process can be elucidated by the hormesis theory which postulates that FR may have a low-dose stimulation high-dose inhibitory effect. Meaning that FR could

provide positive responses when present in small amount (Radak et al., 2008; Radak et al., 2014). As a result, the body's antioxidant network system becomes strong, which minimises the oxidative stress process.

However, not all investigations revealed that antioxidant supplementation hampers exercise-induced activation of redox sensitive signalling pathways (Petersen et al., 2012). There is a plethora of research that has demonstrated the beneficial effects of antioxidant supplementation that show positive outcomes in exercise studies. Furthermore, study also demonstrated that antioxidant supplementation may promotes skeletal muscle mRNA expression of genes involved in mitochondrial biogenesis (Nieman, 2010). Therefore, it could be more beneficial to only consume the antioxidant supplementation during periods of elevated exercise stress. In the next subchapter, only flavanoid, which is quercetin and vitamin C, will be discussed as this type of antioxidant was used for the present study.

2.6.1.1 Quercetin

Recently, flavonoids have extensively been studied among researchers, antioxidant that widely distributed in numerous vegetables and fruits. Apart from numerous vegetables and fruits, flavonoids also can be found in nuts, seeds, spices, grains and beverages (wine, tea and beer) (Kühnau, 1976).

Basic structure of flavonoids consists of 15 carbon atoms arranged in three aromatic rings (ring A, B and C) (**Figure 2.8**) (Galleano et al., 2002; Pietta, 2000; Shashank & Abhay, 2013). The pattern of substitution the A and B rings define individual compound of flavonoids within the class, whereas the level of oxidation and the pattern of substitution of the C ring define the classes between the flavonoids (**Figure 2.8**) (Galleano et al., 2010; Heim et al., 2002; Pietta, 2000; Shashank & Abhay, 2013). There are at least six classes

of flavonoids, those are flavonols, isoflavones, flavones, flavanones, flavonol and anthocyanidins (**Figure 2.8**) (Galleano et al., 2010; Pietta, 2000).

Studies proved antioxidant activity of flavonoids *in vitro* and *in vivo* (Procházková et al., 2011). In vitro, it has been proved that flavonoids have the ability to scavenge FR (superoxide, peroxyl and hydroxyl radical) directly through hydrogen atom donation (Halliwell, 2008), inducing antioxidant enzymes (e.g. glutathione Stransferase, NADPH-quinone oxidoreductase and UDP-glucuronosyl transferase), which are the main defence enzymes against oxidative stress (Nijveldt et al., 2001; Procházková et al., 2011) and inhibit xanthine oxidase that involved in superoxide ion ($O_2^{\bullet-}$) production (Hanasaki et al., 1994).

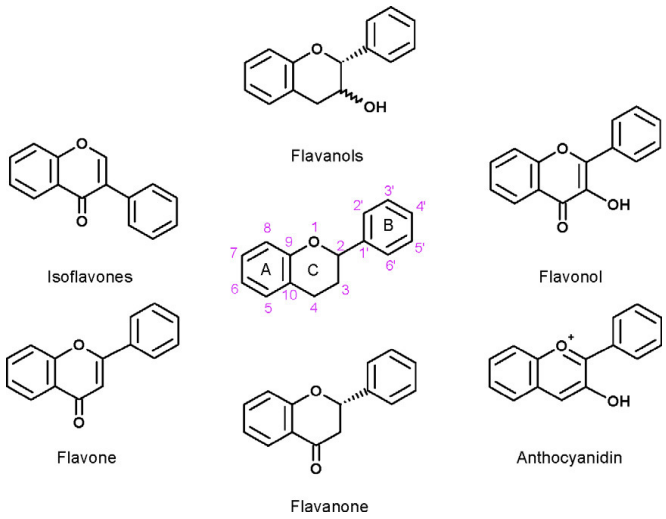


Figure 2.8 Basic structures of flavonoids and different flavonoid subfamilies. Adapted from Galleano et al. (2010).

Quercetin is one of the members in flavonoids family and can be considered the most prominent dietary antioxidants (Boots et al., 2008). Quercetin is mainly found in foods like onions, broccoli, apples, berries, tea and wine (Bohm et al.,1998; Scalbert & Williamson, 2000).

Quercetin has been exposed to be a tremendous *in vitro* antioxidant. Quercetin is acknowledged to be the most powerful scavenger of ROS including superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH·) (Cushnie & Lamb, 2005; Hanasaki et al., 1994) and reactive nitrogen species such as peroxynitrite (ONOO−) (Haenen et al. 1997; Heijnen et al. 2001). In addition, quercetin is identified to possess strong anti-inflammatory, anticarcinogenic, antiviral, neuroprotective psychostimulant and cardioprotective capabilities (Alexander, 2006; Davis et al., 2009b; Harwood et al., 2007; Oršolić et al., 2004; Read, 1995; Utesch et al., 2008).

According to criteria established by the U.S. Food and Drug Administration (FDA) quercetin has GRAS status (generally recognised as safe) (Davis et al., 2009). Supplementation of quercetin does not linked to any harmful adverse effects in both animal and human studies (Harwood et al., 2007; Knab et al., 2011; Utesch et al., 2008).

Based on pharmacokinetic data on doses of pure quercetin that have been used in clinical trials demonstrated that, in human, after ingestion of a 250-500 mg quercetin it can be rapidly increased in plasma within 15-30 minutes, its peak concentration reached at approximately 120-180 minutes, started to decrease around 360 minutes of ingestion and returned to baseline levels after 24 hours (Davis et al., 2009). While, study done by Texier et al. (1998) observed that there was a marked increase of quercetin concentration in plasma 3 hours ingestion of meal that rich in plant products. After 7 hours, the concentration of quercetin began to decline and after 20 hours, it was returned to baseline level.

A previous study regarding the consumption of quercetin in rats exposed that there was an accumulation of quercetin in several organs including the kidneys, lungs, heart, liver and muscle (de Boer et al., 2005). In human subjects, the elimination of quercetin is considered quite slow with a reported half-life ranging from 11 to 28 hours upon absorption of certain amounts of quercetin from food or supplements (Conquer et al.,1998; Manach et al., 2005). This indicates that with repeated quercetin supplementation, human could achieve a considerable level of quercetin in plasma (Hollman et al., 1997; Manach et al., 2005).

Quercetin has been found in contributing beneficial effects on plasma antioxidant and act as effective antioxidants against low-density lipoprotein oxidation (Filipe et al., 2001; Hou et al., 2004). Quercetin has been discovered in improving the antioxidant defence among sarcoidosis (chronic inflammatory disease) patient by increasing in total plasma antioxidant capacity and reducing the blood markers of oxidative stress (Boots et al., 2011). However, some of the human studies failed to detect any differences on oxidative stress biomarkers with daily doses of 1000mg of quercetin supplementation for at least 7 days up to 42 days (Bigelman et al., 2010; Cureton et al., 2009; McAnulty et al., 2008).

Reported by previous findings, simultaneous consumption of quercetin with vitamin C, folic acid and additional flavonoids advances its bioavailability (Harwood et al., 2007; Manach et al., 2005; Moon & Morris, 2007). Antioxidants are chemically converted into its oxidised form when they neutralise the FR. Similar to quercetin, when quercetin act as a free radical scavenger, at the same time, quercetin itself being oxidised into an oquinone/quinonmethide, known as QQ (**Figure 2.9**) (Boots et al. 2008). QQ is highly reactive, however QQ can be recycled back to its parent compound with other antioxidants (ascorbate, glutathione (GSH) and NADH) and it becomes available again to act as

antioxidant (Askari et al., 2012; Boots et al., 2003). An adequate plasma ascorbate level therefore should be maintained when high doses of quercetin are supplemented.

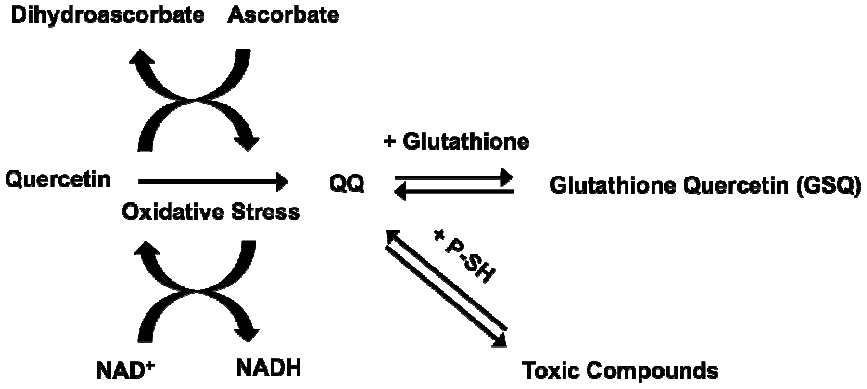


Figure 2.9 Possible reactions of the oxidation product of Quercetin. Adapted from Boots et al. (2008).

Study conducted by McAnulty et al. (2011) support the fact that ingestion of quercetin mixed with vitamin C, isoquercetin, EGCG (catechin), and n-3 fatty acids for 14 days and during 3 days of cycling at 57% W(max) for 3 hours reduced F2-isoprostanes immediately post-exercise from baseline when compared with placebo. However, there was no effect of the chronic supplementation (14 days) on F2-isoprostanes or any antioxidant measure but the acute ingestion (during 3 days of exercise) managed to suppress the post-exercise of F2-isoprostanes. Elevation of plasma metabolites would be expected due to acute ingestion.

McAnulty et al. (2013) also reported that mixed supplementation of resveratrol and quercetin reduced the level of F2-isoprostanes post-exercise, however, plasma-reducing capacity (e.g FRAP, ORAC and TEAC), protein carbonyls, and inflammation (IL-8 and C-reactive protein) were not affected by the supplementation. It could be due to the fact that resveratrol and quercetin act as direct scavengers of peroxynitrite (ONOO–) and other ROS (Olas et al., 2006) thus prevent the membrane-bound arachidonic acid peroxidation,

which is the precursor of F2-isoprostane formation (Morrow & Roberts, 1997). Therefore it was indicated that this mixed supplementation might contribute in attenuation of oxidative stress.

There is a growing body of literature that recognises the importance quercetin's potential to induce mitochondrial biogenesis through peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) (Malaguti et al., 2013), consequently, it would increase oxidative capability and $\dot{V}O_2max$ as well as endurance exercise performance. This is based upon observations in animal study showed that increase in soleus muscle PGC-1α and SIRT1 mRNA, soleus muscle mitochondrial DNA (mtDNA) and treadmill running time until fatigue (~37%) after 1 week of quercetin supplementation (Davis et al., 2009).

Macrae & Mefferd (2006) was the first study investigated the quercetin's effects on endurance performance. This study revealed that the there was a significantly improved in 30km cycling time trial performance (1.7% improvement) after ingestion of quercetin-containing supplement for 6 weeks. Another study by (Nieman et al., 2010) demonstrated that supplementation of 1000mg of quercetin per day for 2 weeks associated with significantly improvement in 12-min treadmill time trial performance and tended to increase in messenger RNA levels of four genes related to mitochondrial biogenesis (PGC-1α, SIRTUIN 1, cytochrome c oxidase, and citrate synthase) and the relative copy number of mtDNA. However, there were few studies failed to prove these findings (Nieman et al. 2007a; Nieman et al. 2007b; Dumke et al. 2009; Nieman et al. 2009).

However, there is still inadequate knowledge of the possible underlying mechanisms in humans. Several exercise studies were conducted with chronic ingestion of quercetin

(Nieman et al. 2007a; Nieman et al. 2007b; Quindry et al. 2008; McAnulty et al. 2008; Abbey & Rankin 2011; McAnulty et al. 2011), unfortunately lack positive of outcomes were discovered in inhibiting oxidative stress. One feasible explanation for the lack of positive outcomes could be due to ingestion of quercetin 10–24 hours before end of exercise, thus plasma quercetin could drop to very low levels (Nieman, 2010). Given the short half-life of quercetin, this period of time may have caused excessive delay in peak plasma quercetin metabolites (Manach & Donovan 2004; Manach et al. 2005; Moon et al., 2008). The short half-life implies that ingestion within 1-2 hours before and during exercise may be more beneficial in reducing oxidative stress (Morillas-Ruiz et al. 2006; Davison & Gleeson 2007; McAnulty et al. 2013).

Collectively, based on these previous works demonstrating that quercetin might have a potential as antioxidant to countermeasure the effects of oxidative damage if taken acutely few hours before the exercise session or during the exercise session.

2.6.1.2 Vitamin C

Vitamin C (ascorbic acid) is water-soluble antioxidants and present in both extracellular fluid and cytosolic compartment of the cell (Evans, 2000). In fluids, vitamin C has the ability to detoxify ROS such $OH^{\bullet}$, $O_2^{\bullet-}$, fatty acid peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) (Finaud et al., 2006) whereas in cytosol, vitamin C contribute in regenerating the active form of vitamin E and GSH after they have reacted with ROS (Ashton et al., 1999; Evans, 2000).

In some mammals, Vitamin C is synthesised in the liver. However, several species including humans are unable to synthesise vitamin C, therefore it must be ingested. Vitamin C primarily comes from citrus fruits such as lemons, limes, grapefruit, oranges,

strawberries and pineapple (García-Closas et al., 2004; Lee & Kader, 2000; Padayatty et al., 2003). Some vegetables that are rich source of Vitamin C include broccoli, cauliflower, cabbage, zucchini, asparagus, celery and lettuce (Lee & Kader, 2000; Padayatty et al., 2003; García-Closas et al., 2004). As a supplement, vitamin C also available in tablet and powder forms in various doses.

Vitamin C is a six-carbon compound similar structure with glucose (**Figure 2.10**). It presents in two active forms: the reduced form known as ascorbic acid and the oxidised form known as dehydroascorbic acid (Alessio et al.,1997). Vitamin C is considered as a good electron donor, therefore it acts as reducing agent. Associated with this property, vitamin C could help the formation of hemoglobin in the red blood cell by absorbing the dietary iron. Most of all, vitamin c is considered the most powerful and outstanding antioxidant, aid in preventing cellular damage and immune system impairment from FR generated by intense aerobic exercise (Evans, 2000).

As a good electron donor, ascorbic acid supplies electrons for enzymes and other electron acceptors. Vitamin C administration has been reported (Tauler et al., 2003) to influence the activity of erythrocyte antioxidant enzymes (catalase and glutathione peroxidase) plasma urate and ascorbate after duathlon competition and short-term recovery. This may indicate that vitamin C and its dietary supplementation play an important role in protecting against the oxidative stress induced by exercise. Athletes who increased their dietary intake of vitamin C may have valuable effects on aerobic capacity, antioxidant status and immunity (Peake, 2003).

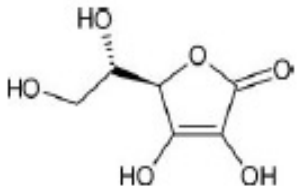


Figure 2.10 Vitamin C or L-ascorbic acid. Adapted from Landete (2013).

Vitamin C has been extensively used as an antioxidant supplement to enhance exercise performance. Several studies (Bloomer et al., 2006; Bryer & Goldfarb, 2006; Goldfarb et al., 2005; Popovic et al., 2015; Zoppi et al., 2006) showed positive effects in regulating redox balance. Supplementation with vitamin C (500mg-3000mg/day) may diminish the production of oxidative stress biomarkers (e.g lipid peroxidation, protein carbonyl) during exercise. It is interesting to note that vitamin C plays a role in directly scavenging free radicals (superoxide, hydroxyl radicals and lipid hydroperoxide), regenerating a-tocopherol (Vitamin E) thus assisting radicals move from lipid to aqueous phase and finally spare GSH during the phase of oxidative stress increased (Carr & Frei, 1999a, 1999b; Meister, 1994; Powers & Jackson, 2008).

The controversy about scientific evidence regarding the influence of vitamin C supplementation on redox status and physical performance has attracted conflicting interpretations among studies. Indeed, several studies have revealed that vitamin C supplementation attenuates oxidative stress, while others have revealed that it does not affect redox status (Choi et al., 2004; Cholewa et al., 2008). Certainly, there are various possible reasons for this discrepancy regarding the effects of vitamin C supplementation on redox status and exercise performance: (1) dose of vitamin C administered, (2) duration of supplementation (3) exercise trials applied, (4) training status of the participants, (5) sampling time point and (6) oxidative stress biomarkers determined.

Recent finding (Paschalis et al., 2016) found that individuals antioxidant level could influence the results of vitamin C supplementation. This recent study demonstrated that supplementation of vitamin C to the group with low level of vitamin C (below 41 µmol/L) decreased the baseline concentration oxidative stress biomarkers (F2-isoprostanes & protein carbonyls) greater than the high vitamin C group (above 71 µmol/L). Furthermore, only low vitamin C group showed increasing in $\dot{V}O_2max$ after vitamin C supplementation. This may indicated that individuals with low baseline level in the oxidant biomarkers likely to exhibit greater percent increases in the biomarkers after exercise and vice versa (Margaritelis et al., 2014)

2.7 SUMMARY AND CONCLUSION

It is clear that there is dose-intensity relationship between exercise and changes in redox balance. Higher volume of exercise intensity and duration appears to be associated with increasing in FR production. In addition, it is clear that hyperthermia also play an important role in inducing the response in redox balance. Nevertheless, the human body is armed with highly effective endogenous antioxidant defence systems. This antioxidant defence will detoxify the FR induced tissue damage for human body to remain in redox balance. However, if FR production exceeds the capacity of the antioxidant system to counteract, oxidative stress and cell damage will occur.

Given that military personnel are easily exposed to oxidative stress due to their vigorous training intensity, we explored the redox balance among military personnel in **Chapter 4**, which is predisposed to heat illness. This topic will be discussed extensively in **Chapter 4** to examine whether heat illness has a relationship with oxidative stress.

Heat stress are suggested to be one of the environmental stress that play a role in inducing oxidative stress, we decided to investigate the redox balance during exercise heat stress with acute antioxidant supplementation in **Chapter 5**. This topic will be explained in more detail in **Chapter 5**.

Given the fact that HSPs productions are intrinsically linked with oxidative stress thus play a role inside (intracellular) and outside the cells (extracellular) under stress condition (exercise heat stress). **Chapter 6** will investigated the effects of acute antioxidant supplementation on intracellular HSPs (muscle cells and peripheral blood mononuclear cells (PBMC)) and extracellular HSPs (plasma) during exercise heat stress.

In summary, overall aims of this thesis is to test the hypothesis that exertional heat illness (EHI) is associated with oxidative stress with examining the redox balance in military recruits undertaking strenuous exercise and to determine whether the acute supplementation can influence the ability of the antioxidant response and HSPs (intracellular & extracellular) during exercise heat stress. By investigating the role of antioxidant supplementation during exercise induced oxidative stress, this thesis hope to learn more about these interactions and the potential benefits of supplementation, thus develop effective methods for improving human health and/or performance in response to oxidative stress.

CHAPTER THREE

GENERAL METHODOLOGY

3.1 INTRODUCTION

This thesis consists of three experimental chapters. The specific design of the first experimental chapter (**Study 1**) will be described in the subsequent chapter (**Chapter 4**), whereas this chapter will describe in details the methods for the second (**Study 2**) and third (**Study 3**) experimental chapters which were mainly performed in some or all of the studies.

3.2 STUDY DESIGN

A double blind, randomised, crossover design was employed for the second (**Study 2**) and third (**Study 3**) studies. Participants performed 3 separate experimental trials. The experimental conditions consisted of three supplementations: Quercetin (Q); Quercetin plus Vitamin C (QC) and; Placebo (P). The minimum washout period between experimental conditions was 14 days.

3.3 SUBJECTS

Participants for second (**Study 2**) and third (**Study 3**) studies were male recreational athletes, aged between 18-35 years, who declared that they were non-smokers and not currently taking mineral or vitamin supplements. Each exercised for at least 2 times per week and for more than 20 minutes per session. This was deemed the minimum cardiorespiratory conditioning to ensure that they should be able to run at 70% $\dot{V}O_2$max for at least 60 minutes in the hot and humid environment (described later in this chapter). Each participant was informed about the experimental design and protocol (**Figure 3.1** and **3.2** respectively) and the possible risks before giving informed written consent to participate. The study was approved by the National Research Ethics Service (NRES) Committee South West - Frenchay with REC reference no.14/SW/0098.

3.4 SUPPLEMENTATION

The supplements are Quercetin (Nature's Best, UK), Vitamin C (Holland & Barrett, UK) and placebo (DR T&T HEALTH, UK LTD). Placebo used in this study was hydroxypropylmethylcellulose (HPMC) vegetarian capsules which 100% natural, contained no preservatives and gelatin. In second (**Study 2**) and third (**Study 3**) studies, both of the supplements were consumed in tablet form. Participants were asked to consume the supplement 14 hours before (9:30pm the night before), 2 hours before (7:30am) and every 20 minutes during the trial.

3.5 PRE-EXPERIMENTAL CONTROL MEASURE

Participants were asked to complete a weighed food record diary for the 72 hours period prior to each experimental trial and were required to replicate the same diet during the 72 hours period prior to each subsequent trial.

They were also asked to abstain from alcohol and caffeine intake and to refrain from strenuous exercise for at least 48 hours prior to each trial, taking a complete rest day during the 24 hours immediately prior to each trial. Participants were asked to abstain from any mineral or vitamin supplements (other than those provided), or any other antioxidant supplements for at least 2 weeks before and during the trials.

Participants were asked to fast from 11:00pm the evening before the experimental trial. However, they were permitted to drink plain water. Prior to each visit, participants consumed 500ml of plain water, approximately 2-hours before exercise. This practice was based on the recommendations of the American College of Sports Medicine's Position Statement on the maintenance of hydration (Sawka et al., 2007).

3.6 EXPERIMENTAL DESIGN

3.6.1 Preliminary measurements:

Prior to the start of the main trials, participants were required to attend the laboratory on two occasions. The first visit included the anthropometric measurements: height and body mass. Following this, participants were fitted with a telemetric heart rate monitor around their chest (Polar FT1, Polar Electro OY, Kempele, Finland) and then participants were required to perform a continuous incremental running protocol until exhaustion on a motorised treadmill (Woodway ELG70, Weiss, Germany). The test protocol was modified from (Taylor et al., 1955). This test was performed in thermoneutral conditions (19.7 ± 0.7°C, 46.3 ± 4.0% relative humidity). After a warm-up for 5 minutes at speed 6km·h^{-1}, the test initiated at a speed of 10km·h^{-1} on a 1% inclination. The speed was increased by 1 km·h^{-1} at the end of each three minute until reaching 13km·h^{-1}, and then inclination was increased by 2% every two minutes. Heart rate and rate of perceived exertion (Borg's Scale, **Appendix A**) were taken during the final minutes of each increment. Only the result during the final minute of each 4-minute increment was taken into account. This was based on the fact that subjects can only reach a steady state of $\dot{V}O_2$ after running for approximately three to four minutes (McArdle et al., 1991). Participants were instructed to run for as long as possible and signal when they felt they could only complete one more minute to allow for a final set of recordings. From the results of these tests, appropriate running intensity used in the main experimental trials has been determined.

3.6.2 Familiarisation trials:

The second visit, participants were required to undergo familiarisation trial to run in the heat at 70% $\dot{V}O_2$max for 60 min (33.2 ± 0.2 °C, 26.7 ± 3.3% relative humidity) prior to the main experimental trials. Tablet of placebo were consumed as the same placebo during the main experimental trials with 3mL.kg-1 body weights of plain water. The aims of this

trial were threefold: (1) to familiarise the participants with the procedures and the physical stress of the exercise to be encountered during the main trials; (2) to ensure that the selected work rate did elicit a relative intensity of 70% $\dot{V}O_2max$; (3) to ensure that the participants could maintain the selected intensity for 60 min after an overnight fast.

The heat environment was produced in a regulated environmental chamber. To raise the ambient temperature till 33°C, 2KW thermostat electric fan heater (Model FH204B) and 2KW convecter heater (Model DL10 STAND) were used, and to maintain the relative humidity of 40%, dehumidifier (Clean Air Optima Cool/Warm mist Humidifier CA-606, Netherlands) was placed within the chamber. Room temperatures and relative humidity were measured throughout the trial by using a wireless hygrometer (Testo 625, United Kingdom).

3.6.3 Main experimental trials:

The general experimental protocol is shown in **Figure 3.2** and the protocol details are described as follows. Participants were randomly assigned by an electronic research randomisation generator to consume either tablets of 1000mg Quercetin (Q), 1000mg Quercetin plus 1000mg vitamin C (QC) or placebo (P) 14 hours before (7:30pm the night before) and 2 hours before (7:30am) exercise, participants were consumed tablets of 500mg Q, QC or P.

Upon arrival at the University of Bath Laboratory at 8:30am, following an overnight fast, nude body mass were recorded (Seca 880, Seca, Hamburg, Germany). Then, participants were asked to insert a sterile rectal thermistor probe (Grant Squirrel 2020, Grant Instruments, Shepreth, UK) to a depth of 10 cm and were fitted with a telemetric heart rate monitor around their chest (Polar FT1, Polar Electro OY, Kempele, Finland).

After that, participants were cannulated with an indwelling cannula (BD Insyte-W, Becton Dickinson, Utah, USA) into an antecubital vein of the participants left arm for blood drawing purposes. The baseline 7ml blood sample was then drawn after a 20-minute stabilisation period with the participant lying supine. Patency of the cannula will be maintained with saline (0.9% sodium chloride, Braun, Melsungen, Germany). For every blood withdrawal, 7mL of blood were collected in a 10mL sterile syringe. Approximately 5mL of saline will be injected into the extension tube after each blood withdrawal to avoid blood clotting.

Starting at 9:30am, participants entered the regulated environmental chamber to begin the warm-up session. Immediately before warm up, room temperature, relative humidity, resting heart rate and core body temperature were recorded. Then, participants began the warm-up session, ran on a motorized treadmill at speed calculated to elicit work rate at 50% $\dot{V}O_2$max on a fixed inclination (Jones & Doust, 1996). At the end of warm-up session, blood sample and expired air were collected, room temperature, relative humidity, heart rate, core body temperature, ratings of perceived exertion (Borg's scale) and thermal discomfort scale (**Appendix B**) were recorded during the last minute of the warm up followed by plain water ingestion of 3ml.kg^{-1} body mass with appropriate tablet (Q, QC or P). Then, the intensity of running increased to 70%$\dot{V}O_2$max of their respective $\dot{V}O_2$max during the trial.

At the intervals of 10 minutes during the trial, room temperature, relative humidity, heart rate, core body temperature, ratings of perceived exertion and thermal discomfort were recorded. Expired air and blood samples were obtained at intervals of 20 minutes during the trial. Following the collection of expired air, participants has been asked to consume 3mL.kg^{-1} body weight of plain water with tablet of either 500mg Q, 500mg Q + 500mg C or P to prevent any adverse effects of dehydration. For the participants who did not complete

the full 60 minutes running, the termination time were recorded and all measurements were taken in the final minute before termination. Post exercise nude body weight was obtained after the subjects have towel-dried themselves. After one-hour post exercise, blood samples, heart rate and rectal temperature were obtained again.

3.6.4 Peripheral Blood Mononuclear Cells (PBMC) Collection & Muscle Biopsy

Study 3 (**Chapter 6**) investigated the effects of acute antioxidant supplementation on intracellular HSPs (muscle cells and peripheral blood mononuclear cells (PBMCs) and extracellular HSPs (plasma) during exercise heat stress. Considering that HSPs productions are intrinsically linked with oxidative stress, two days before (baseline) the start of the main experimental trials, participants were required to attend the second preliminary test which involved PBMC collection and taking a muscle biopsy sample.

Before each muscle biopsy session, 12mL of venous blood samples were collected through venepuncture of a forearm vein into Vacutainer tubes (Nu-Care Products Ltd, Bedfordshire, UK) containing K_2EDTA.

The PBMCs separation started by pipetting Ficoll-Paque Plus solution (3mL) (GE Healthcare, Uppsala, Sweden) were pipetted onto Leucosep tubes (Greiner Bio One, VWR, United Kingdom), then centrifuged for 1 min at 1000 x g. After that, the whole blood (6mL) was added to Leucosep tubes and PBMCs were isolated from whole blood samples by centrifugation for 10min at 1000 x g.

The PBMCs were then aspirated gently into 15mL conical centrifuge tubes (Fisher Scientific) and washed with PBS at 7min at 300 x g. The PBMCs pellet were re-suspended with freezing mix (70% RPMI, 20% FBS, 10% DMSO) and transferred onto Cryovial (Nalgene, Sigma-Aldrich). After that, the PBMCs were frozen in Mr. Frosty™ Freezing Container (ThermoFisher Scientiific, UK) at -80°C for subsequent analysis (HSP70 and

HSC70). All the PBMCs were analysed by Western Blot and the protocol was described thoroughly in **Study 3** (**Chapter 6.2.8**). Plasma collected (~1mL) from PBMCs separation was kept in different eppendorf tube in -80° C for analysis of plasma heat shock protein 72 (eHSP70).

Then, muscle biopsy sample was taken from the vastus lateralis muscle in the thigh under a local anaesthetic for the determination of intramuscular heat shock proteins (HSPs) expression associated with endurance exercise in the heat. The muscle biopsy were taken in an opt out manner, thus allowing participants who are unwilling to provide a muscle biopsy sample to remain eligible to take part in the study. The measurements took place at the University of Bath Laboratories.

A needle biopsy technique (Bergstrom, 1962) was used to obtain muscle tissue from the *vastus lateralis* throughout the series of experiments. The participants were in a semi-supine position. The skin around the sampling site was shaved if necessary and then sterilised by using iodine solution. Following a small incision (≈2-3 mm) in the skin and fascia using a scalpel blade (Swann Morton, UK) and performed under a local anaesthetic (1% Lidocaine; Hameln Pharmaceuticals Ltd., UK), a 5mm gauge Bergstrom biopsy needle (Roberts Surgical Healthcare Ltd., UK) was used to obtain 30-100mg of wet muscle tissue. The biopsy samples were taken proximally to any preceding sampling site and a distance of at least 2.5cm was allowed between each prospective site in order to minimise the effects of an inflammatory response on the muscle (Thienen et al., 2014). An opposite leg was used during the second main experimental trial for each participant, where the use of dominant/non-dominant limbs was counterbalanced between participants.

Once removed from the thigh, each muscle sample were immediately ‘snap-frozen’ by immersion in liquid nitrogen and stored at -80°C pending subsequent analysis for intramuscular heat shock protein (HSP70, HSC70 and HSF-1). All the muscles were

analysed by Western Blot and the protocol was described thoroughly in **Study 3** (**Chapter 6.2.7**)

Two days (post-exercise) following each exercise protocol, a further muscle biopsy sample will be obtained from each participant. Each trial will be separated by at least 7 days from post biopsy to another pre biopsy (Morton et al. 2006). For the second and third trials' the study protocol will be identical to the first trial.

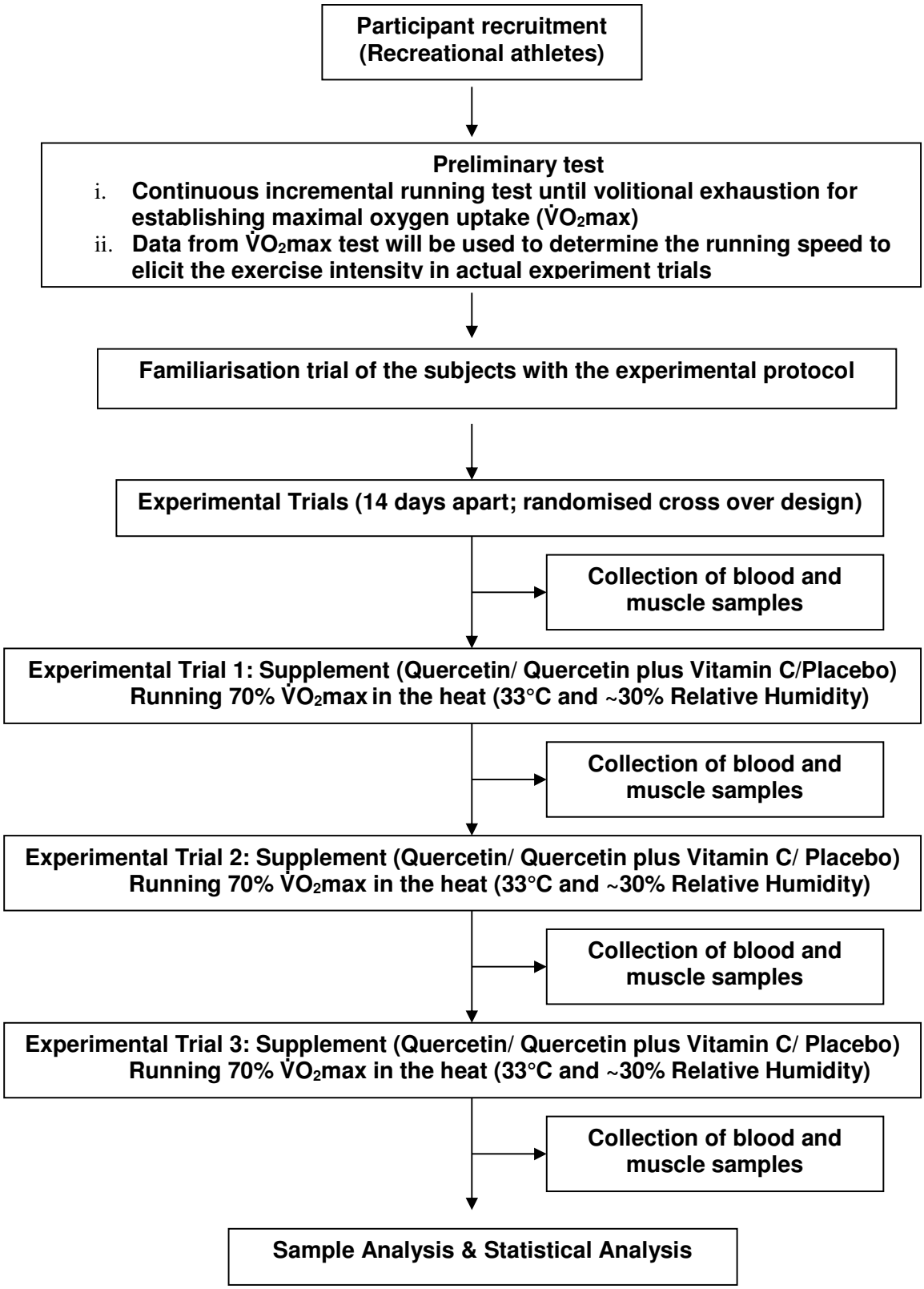
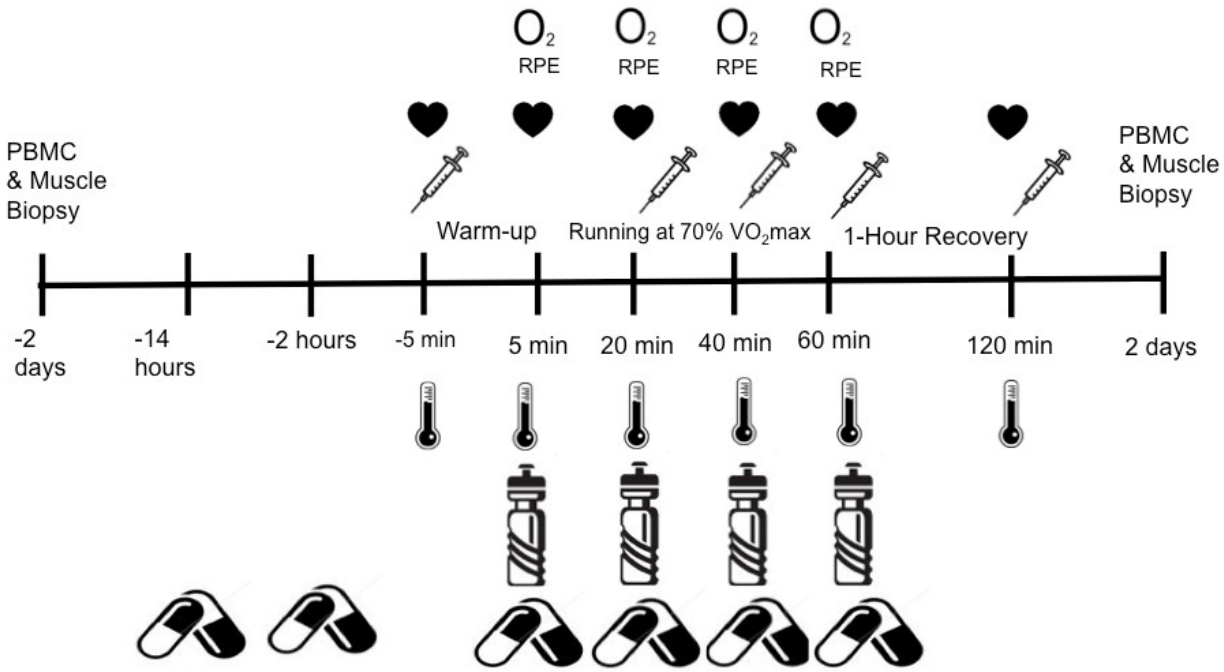


Figure 3.1 General experimental design



RPE: Rating of perceived exertion

O_2 : Expired air collection

: Heart rate

: Blood sampling

: Rectal temperature

: Plain water ingestion

: Antioxidant supplementation

Figure 3.2 General experimental protocol

3.7 ANTHROPOMETRIC MEASUREMENT

All nude body weight (kg) measurements were recorded to the nearest 0.1kg by using an electronic weighing scale (Seca 880, Seca, Hamburg, Germany). While, the height (cm) measurement were recorded to the nearest 0.1cm by using a Harpenden Stadiometer with high-speed veeder-root counter (Holtain Ltd., UK). Subjects stood bare-footed on the weighing machine without clothing that could influence the results when determining the nude body weight. Body Mass Index (BMI) was measured by calculation as follows:-

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height}^2\ (\text{m}^2)}$$

3.8 ANALYSIS OF PHYSIOLOGICAL PARAMETERS

3.8.1 Core Temperature

Core temperature was obtained by measuring the rectal temperature with rectal thermistor probe (Grant Squirrel 2020, Grant Instruments, Shepreth, UK) before warm up, immediately after warm up, at every 10 minutes during the trials, at the end of the time trial and one hour post exercise.

3.8.2 Percentage of body weight changes was calculated as follows:

$$\text{Percentage of body weight changes} = \left[\frac{\text{Post trial weight} - \text{pre trial weight}}{\text{Pre trial weight}} \right] \text{X } 100$$

3.8.3 Physiological Strain Index

Physiological strain index (PSI) was calculated using the following equation (Moran et al., 1998):

$$PSI = 5 \times \frac{Tc_2 \times Tc_1}{39.5 \times Tc_1} + \frac{HR_2 \times HR_1}{180 \times HR_1}$$

where Tc_2 is the post exercise rectal temperature, Tc_1 is the baseline rectal temperature and HR_2 is the post exercise heart rate and HR_1 is the baseline heart rate.

3.8.4 Hydration Assessment

Participants were voiding their bladder to provide a urine sample, which was analysed prior to each trial to ensure they were sufficiently hydrated. Urine was measured for both urine specific gravity (USG; Atago Refractomer, Jencons Pls, Leighton Buzzard, UK) and urine osmolality (Advanced instruments Model 3320, Advanced Instruments Inc, Massachusetts, USA). Participants were considered euhydrated with urine specific gravity ≤1.020 $g \cdot ml^{-1}$ (Armstrong, 2005) and urine osmolality values <900 $mOsmol/kg^{-1}$ (Maughan & Shirreffs, 2008; Peacock et al., 2011; Shirreffs & Maughan, 1998). If the participant was not meet the criteria, the participant was asked to consume a substantial amount of water and the test was repeated again once they ready to urinate.

3.9 BLOOD MEASUREMENT

Ten mL of blood was collected from the participant into the ethylenediamine tetraacetic acid (EDTA) collection tube before warm up, immediately after warm up and every 20 minutes during exercise. Heamoglobin and haematocrit concentrations were analysed by using an automated haematological analyser (Sysmex KX-21N).

Following centrifugation, plasma were separated and stored in 1.5ml aliquots at -80°C for subsequent analysis. Samples were analysed subsequently for ferric reducing ability of plasma (FRAP), protein carbonyl, plasma heat shock protein 72 (eHSP70), plasma heat shock protein 90 (eHSP90α) and plasma quercetin.

3.9.1 Plasma Volume Changes

Changes in plasma volume could affect blood concentrations of biochemical markers (Kargotich et al., 1997) therefore all plasma measurements (FRAP, protein carbonyl, eHSP70 and eHSP90α) were corrected for plasma volume changes. Haemoglobin and haematocrit concentrations were analysed by using an automated haematological analyser (Sysmex KX-21N). Plasma volume changes were calculated using the methods of Dill and Costill (1974) using the following equation:

Protein carbonyl, FRAP, eHSP70 and eHSP90α were corrected for these plasma volume changes using the following equation:

$$\%\Delta PV = \left(\frac{hg_1 \times (1-hct_2)}{hg_2 \times (1-hct_1)} - 1 \right) \times 100$$

3.9.2 Protein Carbonyl

Protein carbonyl a measure of protein oxidation was measured by enzyme-linked-immunosorbent-assay (ELISA) according to the procedures recommended by the manufacturer (Oxiselect, Cell Biolabs, San Diego, USA). Samples and standards (100 µg of 10 µg/ml) were allowed to bind to 96-well protein binding plates in 4°C overnight in duplicate. Dinitrophenol hydrazine (DNPH) working solution (1mg/ml stock solution to 0.04mg/ml DNPH diluent) was added, and plates were incubated for 45 minutes at room temperature in the dark. Plates were blocked 2 hours with blocking solution (5g of blocking reagent in 100mL of PBS) on an orbital shaker at room temperature. A diluted Anti-DNP antibody (1:1000 with 1X blocking solution) was incubated with samples for 60 minutes at room temperature on an orbital shaker, followed by a diluted HRP conjugated secondary antibody (1:1000 with 1X blocking solution), incubated for 60 minutes at room temperature on an orbital shaker. 100µL substrate solution (was added, and plates were left to develop the reaction at room temperature on an orbital shaker for at least 15 minutes (maximum 30 minutes). The enzyme reaction was stopped with stop solution and plates were read at 450nm (620nm as reference wave length). Values were expressed as nanomole per milligram of protein.

3.9.3 Plasma Antioxidant Capacity (FRAP)

Plasma antioxidant capacity was assessed in plasma using the ferric reducing ability of plasma (FRAP) assay established by (Benzie & Strain, 1996). Briefly, standards (0 – 1,000μM ascorbic acid) and samples (10μl/well) were added in triplicate to 96-well flat-bottomed cell culture plates in triplicate. Working reagent (20mM ferric chloride, 160 mM 2,4,6- tripyridyltriazine, 300mM acetate buffer; 300 μl) was warmed to 37°C and added to each well and incubated at room temperature. Absorbance at 593nm was measured after 8 minutes (Spectrostar Nano, BMG Labtech, Ayesbury,UK). Values were determined by linear regression from a seven point standard curve and expressed as μM of antioxidant power relative to ascorbic acid.

3.9.4 Plasma Heat Shock Protein 70 (eHSP70)

In order to decide the best method to analyse eHSP70 in this thesis, we performed a study (Lee et al., 2015) to compare between the 'EKS-715 HSP70 high-sensitivity ELISA' (Enzo life sciences, Lausen, Switzerland) and a new ELISA that has become available ENZ-KIT-101-001 Amp'd® HSP70 high sensitivity ELISA kit (Enzo Lifesciences, Lausen, Switzerland). EKS-715 HSP70 ELISA has been cited by many papers investigating eHSP70 in humans, has a working range between 0.20–12.5 ng·mL^{-1} and a sensitivity of 0.09 ng·mL^{-1} and a new ELISA (ENZ-KIT-101-001 HSP70 Amp'd® ELISA) has a sensitivity to 0.007 ng·mL–1 with a working range of 0.039–5.00 ng·mL^{-1}. Interestingly, our study discovered that ENZ-KIT was superior in detecting resting eHSP70 (1.54±3.27 ng·mL−1; range 0.08 to 14.01 ng·mL−1), with concentrations obtained from 100% of samples compared to 19% with EKS-715 HSP70 ELISA. In addition, eHSP70 after exercise was detected in 6/21 using EKS-715 and 21/21 samples using ENZ-KIT. The details on the method used was described by Lee et al. (2015).

Therefore, based on or published study, the circulating eHSP70 for pre 2 days, pre exercise, end of exercise, post one hour and post 2 days in this thesis were analysed in duplicate by a commercially available ELISA kit which is ENZ-KIT-101-001 Amp'd® HSP70 high sensitivity ELISA kit (Enzo Lifesciences, Lausen, Switzerland). Plasma was selected over serum because it has been shown to produce higher HSP70 concentrations than serum (Whitham & Fortes, 2006).

A minimum dilution of 1:4 (sample to assay diluent) was recommended, however in the present study we found that the 1:4 dilution minimum recommended was not sufficient in some samples which containing eHSP70 above the top standard concentration (5.00 $ng.mL^{-1}$). Therefore, a further analysis was necessary carried out using 1:8, 1:10 and 1:20 dilution step with assay diluent (sodium carbonate) to determine the optimal dilution for each sample, with results multiplied by the this dilution factor in order to give eHSP70 values in $ng.mL^{-1}$.

3.9.5 Plasma Heat Shock Protein (eHSP90α)

The circulating eHSP90α for pre 2 days, pre exercise, end of exercise, post one hour and post 2 days were assessed in duplicate using ELISA kit which is specified for the detection of human HSP90α (AQDI-EKS-895, Enzo Lifesciences, Lausen, Switzerland). Instructions were followed as provided by the manufacturer. Samples were diluted 1:10 with sample diluent provided and a standard curve was used to calculate concentrations in nanogram per millilitre ($ng.mL^{-1}$). eHSP90α were assessed via sample absorbance at 450nm by a microplate reader (Spectrostar Nano, BMG Labtech, Ortenberg, Germany).

3.9.6 Plasma Quercetin

Total plasma quercetin (quercetin and its primary metabolites) was measured using liquid chromatography–tandem mass spectrometry as previously described (Wang & Morris 2005). A matrix matched calibration curve of quercetin was prepared by using blank

human plasma into which increasing concentrations of quercetin was added. Diadzein (D7802, Sigma-Aldrich, UK) was used as an internal standard (IS).

i) Preparation of standard solutions for plasma

A stock solution of 1 mg/ml quercetin was prepared in methanol (MeOH, (Fisher Scientific). Dilution of the stock solution with MeOH yielded working stock solutions at concentrations of 0.02, 0.04, 0.08, 0.4, 1.6, 4.0, 8.0, 12, 16, 32 and 64 µg/ml. A stock solution of the internal standards, diadzein was prepared in MeOH at a concentration of 1 mg/ml and diluted with MeOH to produce a solution with a concentration of 1 µg/ml. Quercetin stock solution (2.5µl) from each concentration (0.02, 0.04, 0.08, 0.4, 1.6, 4.0, 8.0, 12, 16, 32 and 64 µg/ml), 10 µl of diadzein stock solution and 2.5µl formic acid (33015, Sigma-Aldrich) were spiked into 50µl of human plasma.

ii) Enzymatic hydrolysis of quercetin conjugated metabolites

Quercetin conjugates were hydrolysed by incubating 50 µl plasma aliquots with 150µl of 0.2M sodium acetate (pH 4.75), 2.5µl of formic acid, 10µl of diadzein, 5µl of β-glucuronidase (Type HP-2 from Helix pomatia, Sigma-Aldrich), 16µl of Sulfatase (Type H-1 from Helix pomatia, Sigma-Aldrich) for 1 hours at 37°C with continuous shaking (200rpm).

After one-hour incubation, 800µL of MeOH was added to the standards and plasma samples to precipitate plasma proteins; the samples were vortexed for 1min and centrifuged at 13000 rpm for 30min. The supernatant (700µl) was aspirated into new Eppendorf tube and evaporated to dryness using a Speedy Vac (Savant SVC-100H). The dried samples were then reconstituted with 150µL of 10% MeOH, followed by sonication. The samples were filtered using 13mm polyvinylidene Fluoride (PVDF) syringe filters (ESF-PV-13-022, Kinesis, United Kingdom). Thirty microliters (30µl) of the sample was injected during LC-MS analysis.

The LC-MS analysis was conducted using a MaXis HD quadrupole electrospray time-of-flight (ESI-QTOF) mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) operated in ESI negative-ion MS mode. The QTOF was coupled to an Ultimate 3000 UHPLC (Thermo Fisher Scientific, California, USA). The capillary voltage was set to 4500 V, nebulizing gas at 4 bar, drying gas at 12 L/min at 220°C. The TOF scan range was from 75 – 500 mass-to-charge ratio (m/z). Liquid chromatography was performed using an Acquity UPLC BEH C18, 1.7 µM, 2.1 x 50 mm reverse phase column (Waters, Milford, MA, USA) at a flow rate of 0.3 mL/min at 40°C. Mobile phases A and B consisted of H_2O with and methanol, respectively. Gradient elution was carried out with 10% mobile phase B until 2min followed by a linear gradient to 100% B at 5min, keeping 100% B up until 8min, thereafter returned to 10% B until in 12min total run time. The MS instrument was calibrated using a range of sodium formate clusters introduced by switching valve injection during the first minute of each chromatographic run. The mass calibrant solution consisted of 3 parts of 1 M NaOH to 97 parts of 50:50 water:isopropanol with 0.2% formic acid. Quercetin ($C_{15}H_{10}O_7$) and diadzein ($C_{15}H_{10}O_4$) were detected as [M-H]- species with mass-to-charge ratios (m/z) of 301.0354 ± 0.005 m/z and 253.0506 ± 0.005 m/z, respectively. Peaks were integrated and data processed using the Bruker QuantAnalysis Version 4.3 (Bruker Daltonik GmbH, Bremen, Germany).

3.9.6.1 Method Development of Plasma Quercetin Analysis

i) Selection of Enzyme or Non Enzyme Method

Based on developed method (Wang & Morris, 2005) previously, β-glucuronidase and sulfatase were used to measure both the parent and conjugated quercetin in human plasma using LCMS. Therefore, we carried out a test to compare between the sample treated with enzyme and non-treated with enzyme. Based on the presented results (**Table 3.1**), it demonstrated that plasma quercetin concentration was higher when compared to non-treated samples, the enzyme treatment seems to improve the quercetin signal suggesting that some of the conjugated forms are released therefore we decided to treat all the plasma samples using enzymes to hydrolyse the quercetin conjugated metabolites.

Table 3.1 Comparison between enzyme and non-enzyme treated sample for plasma quercetin analysis.

Samples	**Enzyme (μg.L^{-1})**	**Non Enzyme (μg.L^{-1})**
Sample 1	159.5	36.6
Sample 2	154.5	25.9
Sample 3	166.8	28.3
Sample 4	193.4	18.7
Sample 5	180.4	14.2
Sample 6	180.5	19.7
Mean ± SD	172.5 ± 14.8	23.9 ± 8.1

ii) Selection of precipitation method.

The precipitation method is important for the final reconstituted samples, as this would affect the column of the mass-spectrometry (MS), which may disrupt the analysis if the precipitation method did not work. In order to decide the best precipitation method to analyse the plasma quercetin presented in this thesis, we carried out a test to compare the precipitation method by using Methanol, Acetone and Acetonitrile. Based on **Table 3.2**,

Methanol showed the highest plasma quercetin concentration of quercetin compared with Acetone and Acetonitrile, therefore, we decided to use Methanol as our precipitation method.

Table 3.2 Comparison between selected precipitation methods.

Samples	**Methanol** (**µg.L^{-1}**)	**Acetone** (**µg.L^{-1}**)	**Acetonitrile** (**µg.L^{-1}**)
Sample 1	119.4	93.5	82.6
Sample 2	122.3	91.8	79.8
Sample 3	113.4	73.1	73.4
Mean ± SD	118.4 ± 4.5	86.1 ± 11.3	78.6 ± 4.7

iii) Selection of internal standard (IS)

The method described previously (Wang & Morris, 2005) using fisetin as the internal standard (IS). Given that the stability of IS both in standards and sample solution should be considered for the analysis, therefore, we tested the internal standard fisetin (F4043, Sigma-Aldrich, UK) comparing between samples with enzyme treated and non-treated. Interestingly, we discovered that fisetin showed very high peak with samples treated with enzyme (**Figure 3.3**). It seems that the fisetin was being deglucorinated or desulfonated by the enzymes, which is causing this high background. Based on finding from Chen et al. (2009), we decided to use diadzein instead of fisetin. **Figure 3.4** revealed that it seems much less variation between the enzyme treated and non-treated peak areas for the diadzein as IS and the peak is also more prominent (result not shown). Therefore, based on this finding and previous study (Chen et al., 2009), diadzein has been chosen as the IS.

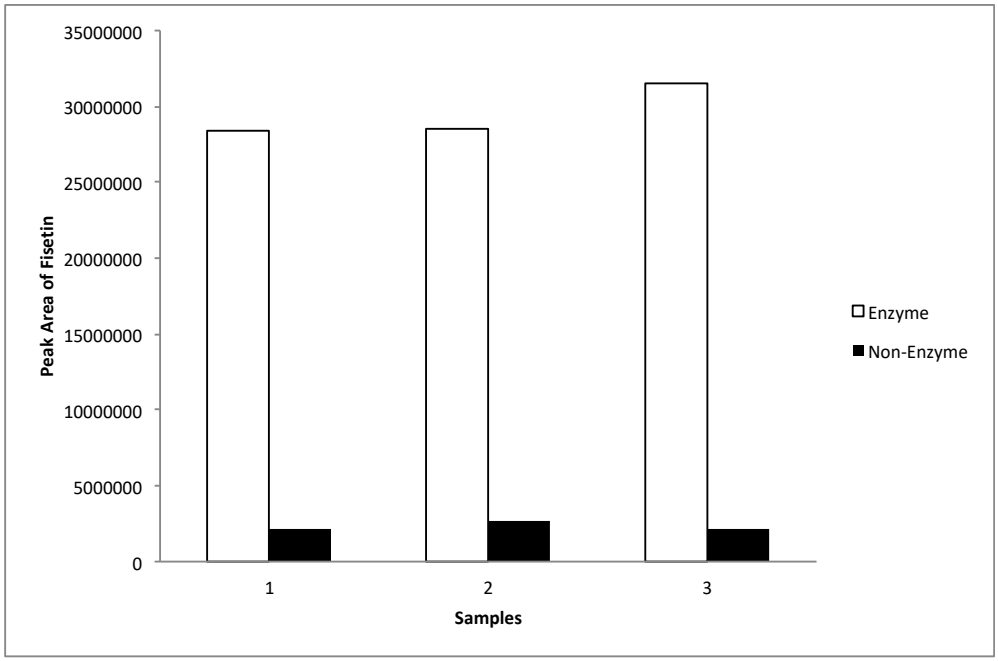


Figure 3.3 Comparison between samples treated with enzyme and non-enzyme using fisetin as internal standard.

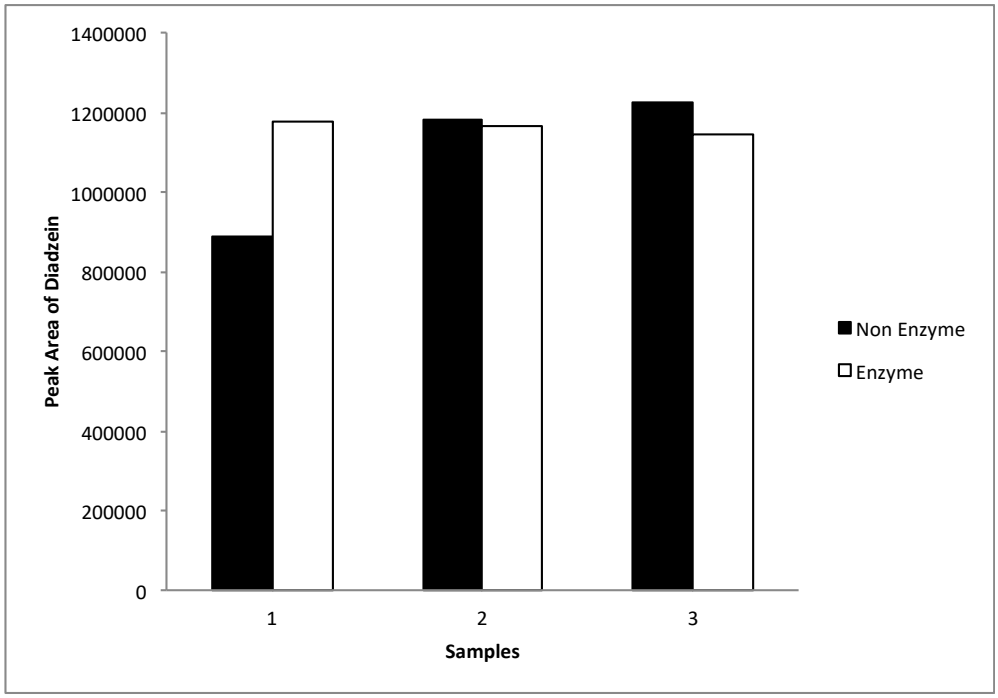


Figure 3.4 Comparison between samples treated with enzyme and non-enzyme using diadzein as internal standard.

iv) Selection of filters

Type of filter also play an important role in analyzing large batch of samples to avoid blockage of MS. Therefore we tested 3 different types of filters: Polyestersulfone (PES), Polyvinylidene Flouride (PVDF) and Nylon supplied by Kinesis (UK). Finding (**Figure 3.5**) revealed that that filtering with PES and Nylon filters removed all of the quercetin and the PVDF membranes results in about a 50% loss however, we injected 30µL of sample instead of 10µL to compensate for these losses.

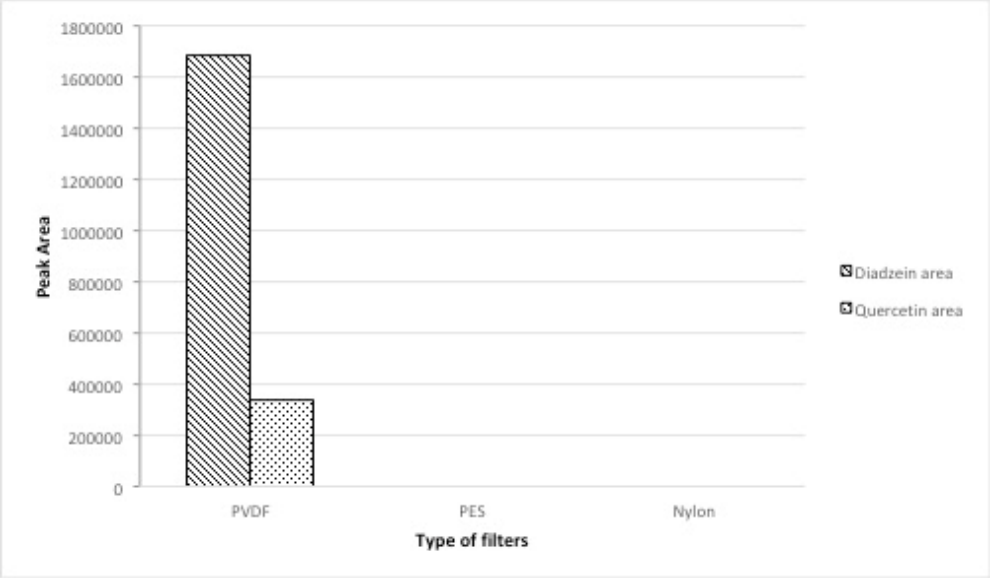


Figure 3.5 Comparison between types of filters: Polyestersulfone (PES), Polyvinylidene Flouride (PVDF) and Nylon.

v) Removal of ascorbic acid from the samples and standards

Based on the established method (Wang & Morris, 2005), the objective for the addition of ascorbic acid (AA) was to adjust the pH of the samples. Interestingly, we discovered that it looks like the peak area of quercetin was better without the AA. The peaks shape is dramatically different, with AA had some tailing. Also, the peak area with AA was about 25% less compared to the without AA. Therefore, we decided to remove the addition of AA completely for all the plasma quercetin analysis.

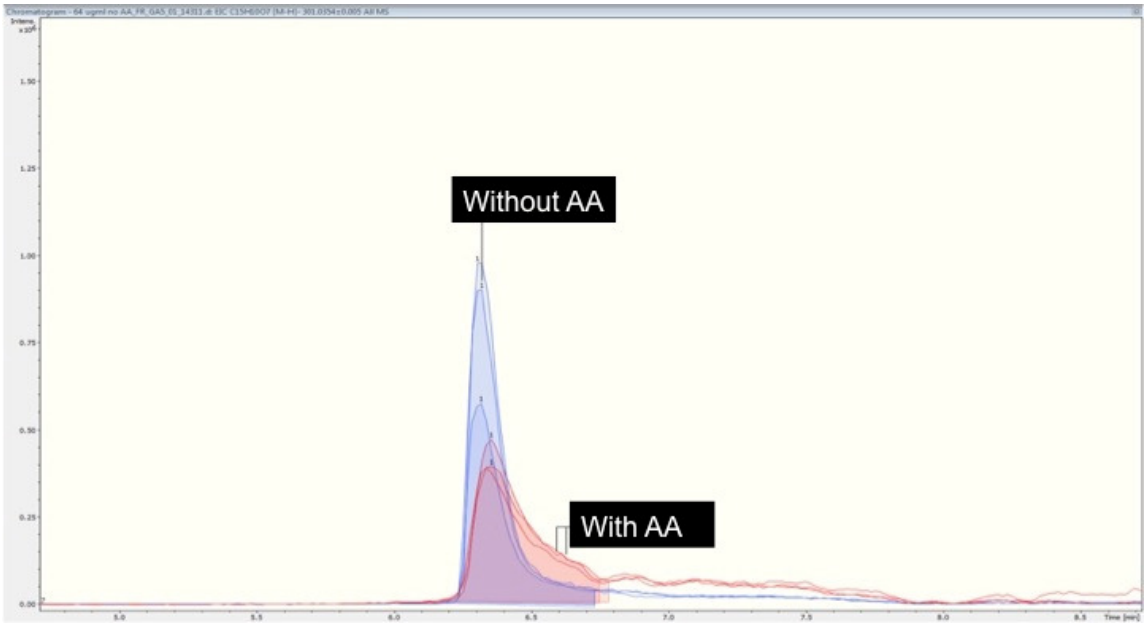


Figure 3.6 Comparison the peak area of quercetin treated with ascorbic acid and without ascorbic acid.

Collectively, based on the series of plasma quercetin method development, we successfully developed the best and suitable method for study in this thesis as described in subchapter **3.9.6**

3.10 RELIABILITY MEASUREMENTS

The coefficients of variation for each blood parameter are shown in **Table 3.3**. Intra-assay coefficient of variation were calculated as follows:

CV % = (Mean of the standard deviations of the duplicates / Grand mean of the duplicates) **X** 100

Table 3.3 Intra-assay coefficients of variation (CV%) for all blood parameter analysis

Assay	Units	Mean Concentration	Number (n)	CV (%)
Bichichonic acid	uL	15.3	24	3.7
Protein Carbonyl	nmol/mg	1.309	24	4.4
FRAP	uM	414.8	30	3.8
Lipid peroxidation	nmol/ml	19.13	26	5.4
eHsp70	$ng.mL^{-1}$	0.457	26	9.2
eHsp90α	$ng.mL^{-1}$	0.702	32	4.1

CHAPTER 4

Study 1: Exertional Heat Illness during Physical Training in The Military: Possible Links to Redox Balance

4.1 INTRODUCTION

Military training sometimes involves prolonged exercise in extreme environmental or ambient conditions, such as hot (> 30°C) or cold (< 0°C) temperatures (Askew, 1993). It is well documented that strenuous extreme environments are associated with high levels of physiological and psychological stress (Acevedo & Ekkekakis, 2001; Bolmont et al., 2000; Gleeson, 2000). For example, the energy demands of undertaking tasks in extreme environments is usually greater compared to less demanding environments for many reasons, such as the ambient conditions (e.g., temperature and humidity), uneven terrain, and the weight of clothing or equipment carried (Askew, 1995). Many aspects of military training are undertaken in hot environmental conditions and the energy expenditure of tasks performed in these settings is increased by the additional work of ventilation and increased sweat-gland activity (Askew, 1995). There was an approximately 10% increase in the energy requirements of work at 38°C compared with the same amount of work at 30°C (Consolazio, 1963).

When undertaking physical work in hot environments there is a large and prolonged production of metabolic heat and as a result individuals operating in these environments are at a greater risk of serious heat illness (Carter et al., 2005). This condition can classified as being “classic” or “exertional” heat illness, with “classic” heat illness typically observed in sick and compromised populations at rest, and “exertional” heat illness primarily observed in healthy and physically fit populations during exercise (Carter et al. 2005). Exertional heat illness is characterised by heat cramps, heat syncope, heat exhaustion, heat stroke, and exertional hyponatremia (Binkley et al. 2002). Less initial and

less severe characteristics of exertion heat illness include negative energy balance, dehydration, impaired thermoregulation, ketosis, altered acid-base and electrolyte balance, depleted muscle glycogen stores, impaired fine motor coordination, and reduced work capacity (Askew, 1993). The clinical presentation of EHI is associated with systemic inflammation, multiple organ dysfunction, and disseminated intravascular coagulation along with high body core temperature, severe physical exhaustion and some loss of consciousness (Bouchama, 2002). It is unclear whether redox balance is another key hallmark of exertional heat illness.

Redox imbalance (i.e., an alteration between states of reduction and oxidation), often referred to as oxidative stress, is a common during and following very demanding exercise. Oxidative stress as the imbalance between production of reactive oxygen species (ROS) and our antioxidant defences, in favour of the former, leading to a disruption of redox signalling and control and/or molecular damage, such as oxidation of proteins, lipids and DNA (Sies & Jones, 2007). In general, the human body has adequate antioxidant defences system to protect tissues against the production of ROS under normal physiological conditions (Birben et al., 2012; Gomes et al., 2012). At rest and possibly low to moderate intensity exercise, these antioxidant defence systems maintain homeostasis for normal cell functions (Gomes et al., 2012). However, when there is an excessive production of ROS, for example during prolonged or vigorous intensity exercise the result can be extensive cell and tissue damage (Ji, 1995). For example, it has been shown that the sustained training load during the last 4 weeks of 8 weeks basic military training led to oxidative stress observable both at rest and after submaximal exercise (Tanskanen et al., 2011)

Since ROS production is a by-product of cellular respiration, it has been suggested that energy expenditure may have an impact on oxidative stress. High metabolic rate is alleged

to be one of the major source of oxidative stress that accompanied military recruits who work in these extreme environments. High amounts of physical exertion causes oxygen consumption to rise several fold. Localized oxygen consumption at certain tissue sites such as skeletal muscle mitochondria can be 10-40 times higher than resting rates (Singh, 1992), leading to a numbers of oxygen being incompletely reduced. Consequently, heightening the production of superoxide radicals (O^{2-}), which further reduced to H_2O_2, hydroxy radicals ($OH^{\bullet}$) and eventually to water (King et al.,1993). However, this notion changed as Boveris & Chance (1973) discovered that leaking of ROS only occurs during State 4 respiration, which happens with low oxygen uptake and adenosine triphosphate (ATP) production but high membrane potential but did not occur during State 3 respiration which involves high oxygen uptake and high ATP production but low membrane potential. Therefore, it may indicate that there are more extramitochondrial sources of ROS during exercise such as ischemia reperfusion phenomenon (Di Meo & Venditti, 2001), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Powers et al., 2011), neutrophils inflammatory response (Pyne, 1994), autooxidation of catecholamines (Ji & Leichtweis, 1997) and haemoglobin oxidation (Cooper et al., 2002).

Previous investigations revealed that ambient temperature might influence the exercise-induced oxidative stress response. McAnulty et al. (2005) demonstrated that treadmill running produced significant core temperature elevations (39.5°C) and elicited a significant increase in lipid peroxidation (show by measurements of plasma F2-isoprostanes) when compared to neutral environment (25°C). Similarly to Quindry et al. (2013), found that 1 hour cycling at 60% Wmax and associated recovery in a warm environment (33°C) induced elevation of ferric-reducing ability of plasma (FRAP), Trolox-equivalent antioxidant capacity (TEAC) and lipid hydroperoxides but these responses did not observed at comparable exercise performed at cold (7°C), and room-temperature environments (20°C).

Adachi et al. (2009) explored a possible linkage between oxidative stress and heat, and convincingly propose that oxidative stress could be a crucial adverse factor in boosting the severity of heat illnesses including heat syncope, heat exhaustion, heat cramps and heat stroke.

Considering the dose response relationship between exercise intensity and duration with reactive oxygen species production, and the likelihood of military training being undertaken in extreme environments, there are limited scientific investigations over a possible role for redox balance being implicated in exertional heat illness. Therefore, the purpose of this study is to explore whether aspects of military recruits suspected as having exertional heat illness, exhibit greater disturbances in redox homeostasis during demanding exercise, compared to military recruits exhibiting no signs of heat illness.

4.2 METHODS

4.2.1 Participants

45 Parachute Regiment Trainees from the British Army were examined in this study which was approved by the Ministry of Defence Research Ethics Committee (MODREC Protocol No: 0911/236 approved on 15 May 2009) and endorsed by the University of Bath's Research Ethics Approval Committee for Health (REACH).

4.2.2 Study design

4.2.2.1 Preliminary measurements

Prior to the Loaded March (LM) (i.e., recruits carrying a 20-kg external load ~110 min while marching) and a Log Race (LR) (i.e., recruits carried a 90-kg log ~20 min running) events, each participant's age, height (Seca Leicester Stadiometer; Hamburg, Germany), body mass (Alpha 770, Seca) and skinfold thickness at four sites (Durnin & Womersley, 1974) was recorded and percentage body fat estimated (Siri, 1956). Maximal oxygen uptake ($\dot{V}O_2max$) was estimated by each participant's 'best effort' 1.5 mile run time, existing

physical training records (Kline et al., 1987). While for those 1.5 mile run time was not available, V̇O2max was estimated from age, gender and anthropometric data (Jackson et al., 1990)

A day before Day 1, at ~05:00 participants voided their bladder and shortly afterwards, nude body mass was recorded. From 18:00 onwards, preliminary anthropometric measurements were taken. At ~22:00, each participant was asked to ingest a pre-coded radio-telemetry pill (CorTemp, HQ Inc, Palmetto, FL) for subsequent body core temperature assessment.

Day 1

On waking (~05:00), a fasted 18 ml venous blood sample was drawn from a forearm vein. Participants voided their bladder and nude body mass was recorded. Each then put on a Polar Team heart rate monitor strap (Polar Team System; Polar Electro Oy, Kempele, Finland) and a CorTemp logger in preparation for the Loaded March. Participants consumed breakfast ~0700. The LM event began at ~09:00. Participants march/ran as a squad, carrying a 20-kg external load, covering 10-miles across undulating terrain in ≤110 min. The mean ambient temperature for LM event was 14.8 ± 2.48°C with relative humidity 71.3 ± 20.7 %. If participants were withdrawn from the event, for whatever reason, a member of the project team collected a post-event blood sample when the medical support team indicated that it was safe to do so. Samples were collected from all other participants within 30 min of completion of the loaded march. At ~22:00 participants were asked to ingest a further calibrated and coded radio-telemetry pill.

Day 2

On waking (~0500), a further fasted 18 ml venous blood sample was be drawn from a forearm vein and nude body mass recorded. Again, each participant then put on a Polar

Team heart rate monitor strap and a CorTemp logger in preparation for the Log Race (LR) event. Following breakfast, participants completed a warm-up procedure by a member of the Physical Training (PT) staff. The main LR event began at ~0900. Briefly, in teams of 6-8, participants carried a 90-kg log across 3-km of undulating terrain in ~20-min. The mean ambient temperature for LR event was 14.8 ± 4.08°C with relative humidity 63.5 ± 19.8 %. Again, if a participant withdrew from the event, either voluntarily or by collapsing on exertion, the project team collected a post-event blood sample when the medical support team indicated. The medical team recorded the level of consciousness (Glasgow Coma Scale; Teasdale & Jennett, 1974) of any casualties, as soon as is reasonably practical. Samples were collected from all other participants within 15-min of completion of the LR.

Individuals who suspected having exertional heat illness during the loaded march were identified as LM-EHI. While individuals who suspected having exertional heat illness during the log race, were identified as LR-EHI. Furthermore, individual, who successfully finished both LM and LR event, were treated as controls (CON) for comparative purposes (**Figure 4.1**).

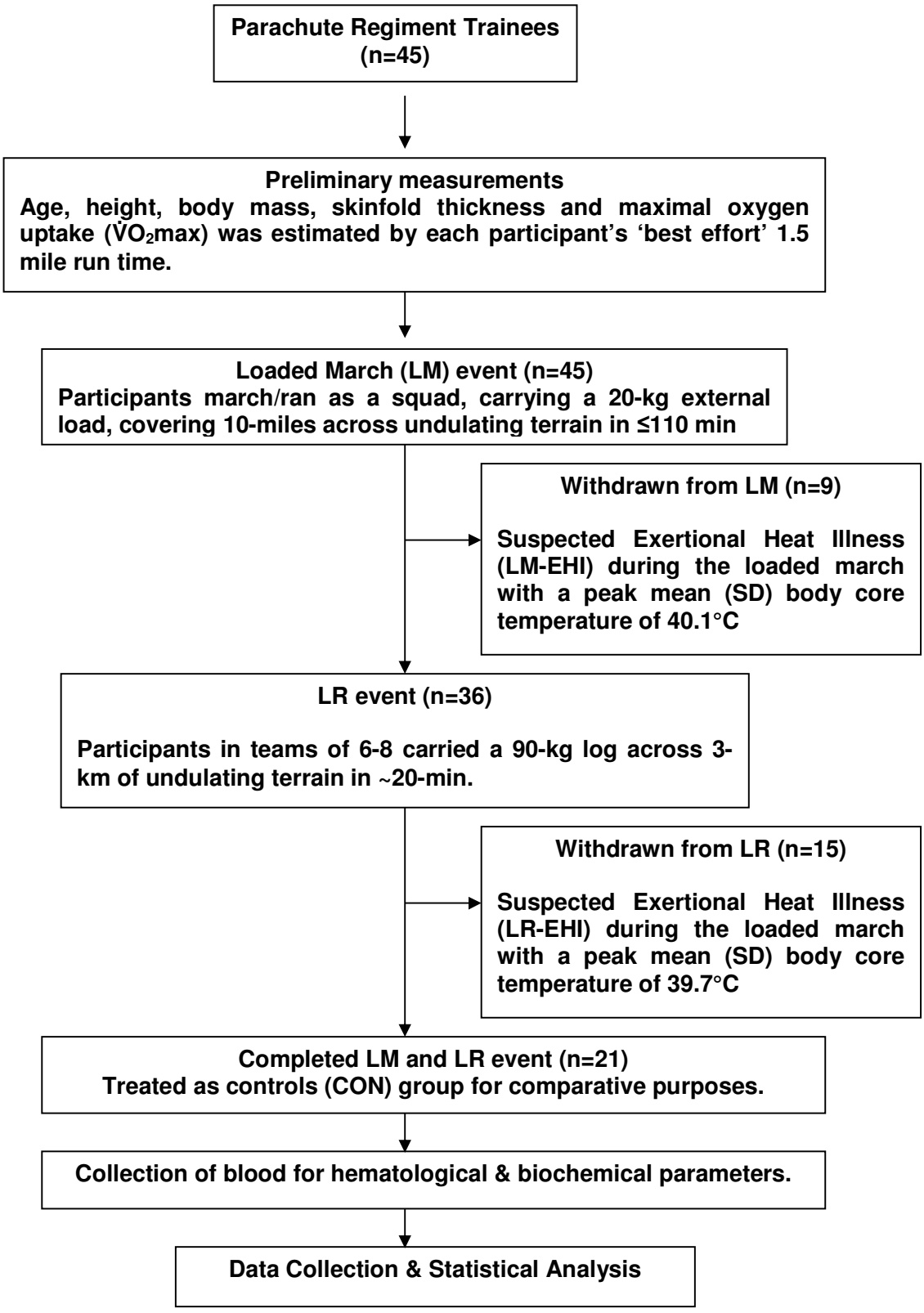


Figure 4.1 Study design

4.2.3 Blood Sampling.

Venous blood samples were collected through venepuncture of forearm vein into Vacutainer tubes (Becton Dickinson, Oxford UK) containing K3EDTA (14ml). Approximately 2ml of EDTA plasma was separated using sterile pipette tips and aliquoted into pyrogen-free glass tubes (Cape Cod International Inc., Liverpool). Samples were then stored at -80°C and subsequently transported on dry ice (compressed CO_2) to the University of Bath and stored at -80°C until subsequent analysis.

4.2.4 Analytical Methods

This study decided to analyse plasma antioxidant capacity, lipid peroxidation and protein carbonyl for LR-EHI during loaded march event for comparative purpose.

4.2.4.1 Plasma Antioxidant Capacity.

Plasma antioxidant capacity was assessed in plasma using the ferric reducing ability of plasma (FRAP) assay established by (Benzie & Strain, 1996). Briefly, standards (0 – 1,000 µM ascorbic acid) and samples (10µl/well) were added in triplicate to 96-well flat-bottomed cell culture plates in triplicate. Working reagent (20 mM ferric chloride, 160 mM 2,4,6-tripyridyltriazine, 300 mM acetate buffer; 300µl) was warmed to 37°C and added to each well and incubated at room temperature. Absorbance at 593nm was measured after 8 minutes (Spectrostar Nano, BMG Labtech, Ayesbury,UK). Values were determined by linear regression from a seven-point standard curve and expressed as µM of antioxidant power relative to ascorbic acid. Assays were undertaken on two separate occasions (Week 1 and Week 2) and data averaged. There were positive correlation between the Week 1 and Week 2 (Pre LM, $r= 0.775$ $p = <0.01$; Post LM, $r= 0.932$ $p = <0.01$; Pre LR, $r= 0.716$ $p = <0.01$; Post LR, $r= 0.846$ $p = <0.01$) indicating that assays performed repeatedly was reliable.

4.2.4.2 Lipid Peroxidation.

Lipid peroxides were assayed in plasma using a modification of the method by el-Saadani et al., (1989). Samples, positive (1:1,000 hydrogen peroxide) and negative controls (distilled water) were added in trilpicate to 96-well plates in triplicate. Working reagent (0.2 M potassium phosphate, 0.12M potassium iodide, 2 g/l Triton-X, 0.1 g/l benzylalkonium chloride, 10μM ammonium molybdate; 100μl) was added, and plates were incubated at room temperature in the dark for 30 min. Absorbance at 365nm was measured (plate reader details). Lipid peroxide concentration was calculated using the Beer-Lambert-Law with an extinction coefficient of 24,600. Values were expressed as nmol/ml plasma. Assays were undertaken on two separate occasions (Week 1 and Week 2) and data averaged. There were a good correlation between the Week 1 and Week 2 (Pre LM, r= 0.945 p = <0.01; Post LM, r= 0.913 p = <0.01; Pre LR, r= 0.847 p = <0.01; Post LR, r= 0.946 p = <0.01) indicating that assays performed repeatedly was reliable.

4.2.4.3 Plasma Protein Concentration

The total concentration of protein in blood plasma was analysed using the bicinchoninic acid (BCA) method as described by (Smith et al., 1985). Samples were diluted to 1:100 dilutions. 25μL of nondiluted standards were added onto the plate in triplicate followed by adding of 25μL of diluted sample. Then, 200μL of working reagent were added to each well. The plate was incubated at 37°C for 30 minutes. Plates were read at 490nm.

4.2.4.4 Plasma Protein Carbonyl Concentration

Protein carbonyl a measure of protein oxidation was measured by enzyme-linked-immunosorbent-assay (ELISA) according to the procedures recommended by the manufacturer (Oxiselect, Cell Biolabs, San Diego, USA). Samples and standards (100μg of 10 μg/ ml) were allowed to bind to 96-well protein binding plates in 4°C overnight in duplicate. Dinitrophenol hydrazine (DNPH) working solution (1mg/ml stock solution to

0.04mg/ml DNPH diluent) was added, and plates were incubated for 45 minutes at room temperature in the dark. Plates were blocked 2 hours with blocking solution (5g of blocking reagent in 100mL of PBS) on an orbital shaker at room temperature. A diluted Anti-DNP antibody (1:1000 with 1X blocking solution) was incubated with samples for 60 minutes at room temperature on an orbital shaker, followed by a diluted HRP conjugated secondary antibody (1:1000 with 1X blocking solution), incubated for 60 minutes at room temperature on an orbital shaker. 100µL substrate solution (was added, and plates were left to develop the reaction at room temperature on an orbital shaker for at least 15 minutes (maximum 30 minutes). The enzyme reaction was stopped with stop solution and plates were read at 450nm (620 nm as reference wave length). Values were expressed as nanomole per milligram of protein.

4.2.5 Data Analyses

All statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS version 22). One-way ANOVA was used to determine the differences of the physical characteristics variables by groups. Two-way ANOVA with repeated measures was used to analyse all variables. Where sphericity was broken, P values were corrected for by using the Greenhouse-Geisser method. Rate of rise core body temperature was measured by using independent T-Test to determine the differences between groups. Pearson's correlation analysis was performed to analyse relationship between two repeated assays (Week 1 and Week 2) for plasma antioxidant capacity and lipid peroxides. All the statistical significance was accepted at $p < 0.05$. All data were expressed as means ± standard deviation (SD).

4.3 RESULTS

Nine (n=9) individuals were identified as having suspected Exertional Heat Illness during the loaded march, with a peak mean (SD) body core temperature of 40.1 (0.5) °C. Fifteen (n=15) individuals identified as having suspected exertional heat illness during the log race, with a peak mean (SD) body core temperature of 39.7 (0.5) °C. A further twenty-one (n=21), which successfully finished both LM and LR event, were treated as controls (CON) for comparative purposes. Exertional heat illness was defined as an individual who becomes incapacitated as a result of a rise in core body temperature (Tc ≥39°C) during a physical exertion, accompanied by some loss of consciousness (Bilzon et al. 2012).

4.3.1 Physiological characteristics of participants

The mean (SD) descriptive characteristics of the CON, LM and LR groups are presented in **Table 4.1**. Control group took part in both the loaded march and the log race. There were no significant differences between groups in any of the variables presented.

Table 4.1 Participants mean (SD) physical characteristics by group.

	Control	Loaded March Heat Illness	Log Race Heat Illness	*P* value between groups
Age	22 (2)	24 (4)	23 (3)	*p = 0.093*
Height (m)	1.78 (0.07)	1.76 (0.06)	1.79 (0.07)	*p = 0.701*
Body Mass (kg)	76.7 (6.9)	76.1 (5.7)	80.1 (8.1)	*p = 0.283*
BMI ($kg \cdot m^2$)	24.2 (2)	24.6 (1.6)	24.9 (2.1)	*p = 0.591*
Body Fat (%)	14.9 (3)	13.9 (2.6)	13.9 (2.8)	*p = 0.553*
1.5 mile run time (sec)	540.3 (37.3)	537.6 (22.6)	549.7 (28.5)	*p = 0.591*
Predicted $\dot{V}O_2max$ (ml/kg/min)	57.0 (3.9)	56.7 (3.5)	56.3 (2.8)	*p = 0.853*

4.3.2 Core Body Temperature

Core body temperature was significantly increased throughout the LM event for both EHI and CON groups $F(2.13,32)= 59.07$, $p<0.001$ **(Figure 4.2A)**. However there was no significant interaction effect between groups on core body temperature $F(1,15)= 2.756$, $p=0.118$. Mean (SD) peak body temperature for the control group at the end of the loaded march was 39.8ºC (0.6) while for EHI 40.1ºC (0.5).

Similar to LR event, there was a significant increase in core body temperature in both EHI and CON groups $F(17,54.1)= 246.07$, $p<0.001$ **(Figure 4.2B)**. However there was no significant interaction between groups on core body temperature $F(1, 32)= 2.788$, $p=0.105$. Mean (SD) peak body temperature for CON at the end of the LR event was 39.4ºC (0.6) while for EHI 39.7.1ºC (0.5).

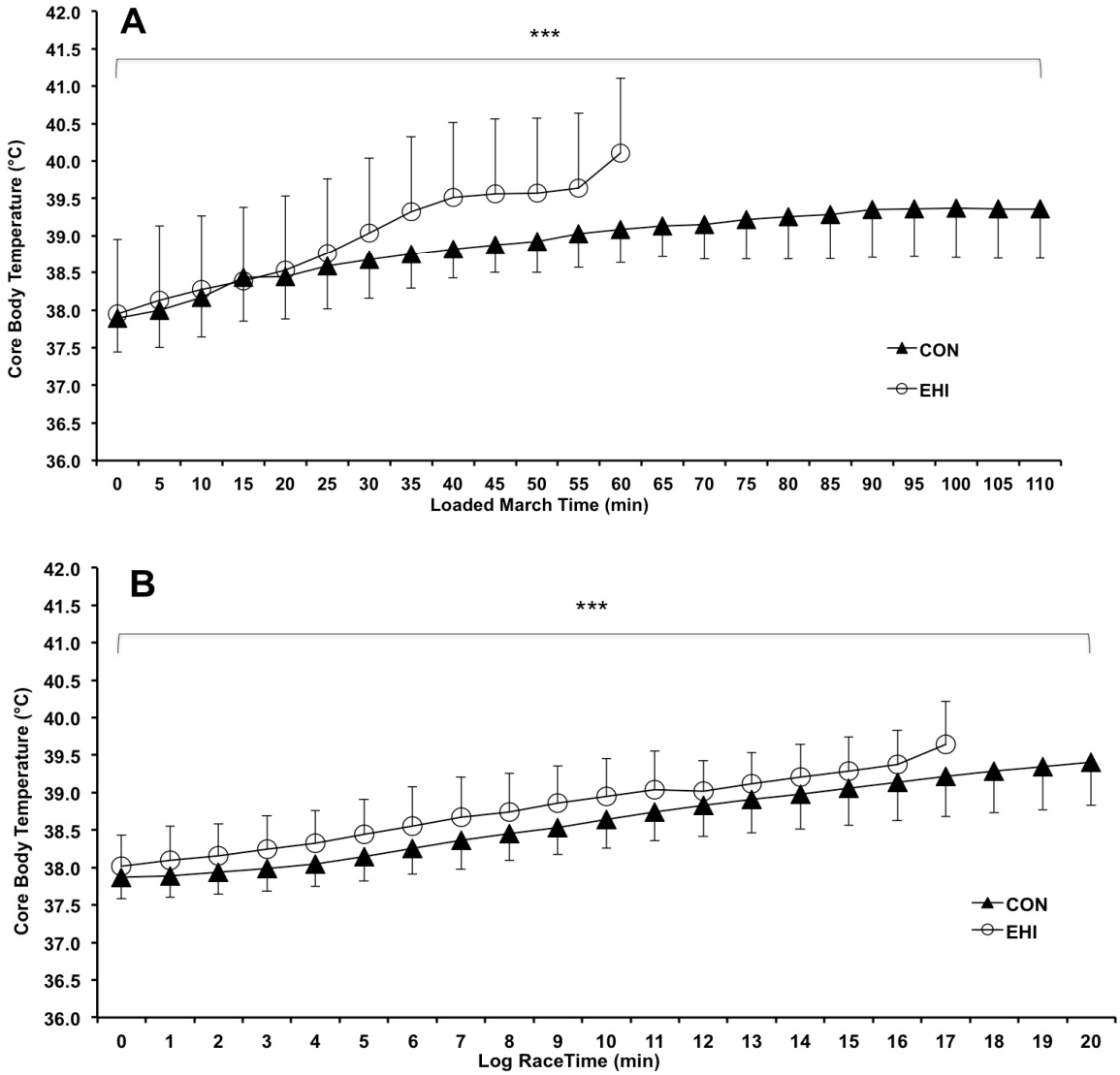


Figure 4.2: Mean (SD) core body temperature during the loaded march and log race in individuals suspected as having exertional heat illness vs. controls. **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).

*** significantly different from respective pre values ($p < 0.001$)

Rate of rise of core body temperature

The rate of rise of core body temperature was not significantly difference F(1,28)= 4.091, p=0.053 between EHI (0.031 (0.011) ºC.min-1) compared to CON group (0.024 (0.009 ºC.min-1) during LM event (**Figure 4.3A)**. However, the rate of rise of core body temperature was significantly greater F(1,32)= 6.854, p=0.013 for EHI (0.1 (0.032) ºC.min-1) compared to CON group (0.076 (0.02 ºC.min-1) during LR event **(Figure 4.3B)**.

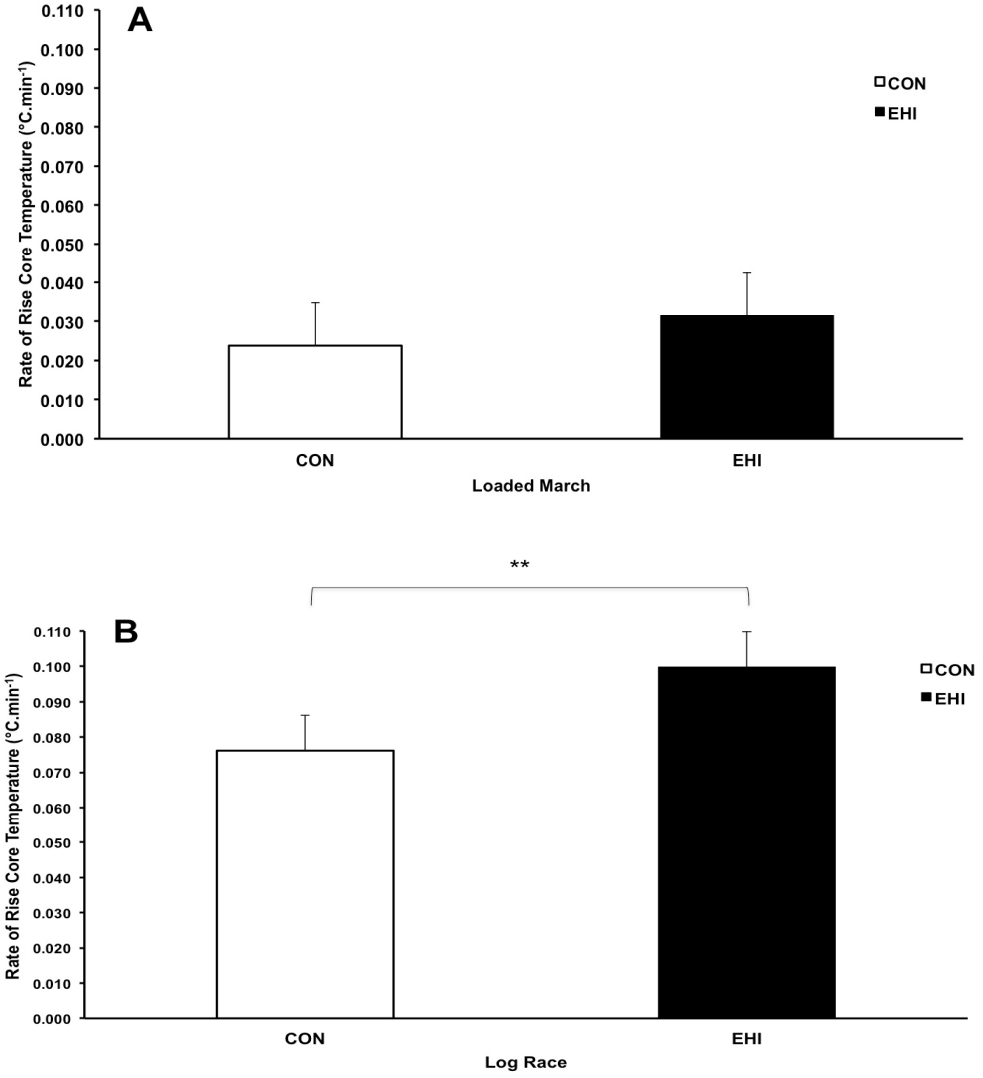


Figure 4.3: Mean (SD) rate of rise core temperature (ºC.min-1). **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).

** significantly different between group ($p < 0.01$).

4.3.3 Heart Rate

All individuals exhibited an increase in heart rate (HR) during both LM $F(2.85, 28.5)= 29.7$, $p<0.001$ (**Figure 4.4A**) and LR $F(3.07, 24.5)= 70.54$, p,0.001 (**Figure 4.4B**) events compared to rest. There were no significance differences between EHI and CON group during both LM $F(1,10)= 1.147$, $p=0.309$ and LR $F(1, 8)= 4.019$, $p=0.08$ event on the heart rate (b.min-1) responses. Lacking in significant difference between EHI and CON during both LM and LR event implied that all participants received similar cardiovascular strain. Mean heart rate at the end of LM event for EHI and CON in the present study were 184±14, which is 93.8% of their maximum HR and 188±10 beats.min-1, which is 94.9% of their maximum HR respectively. Whilst, Mean heart rate at the end of LR event for EHI and CON were 198±1, which is 100% of their maximum HR and 185±6 beats.min-1, which is 93.9% of their maximum HR respectively.

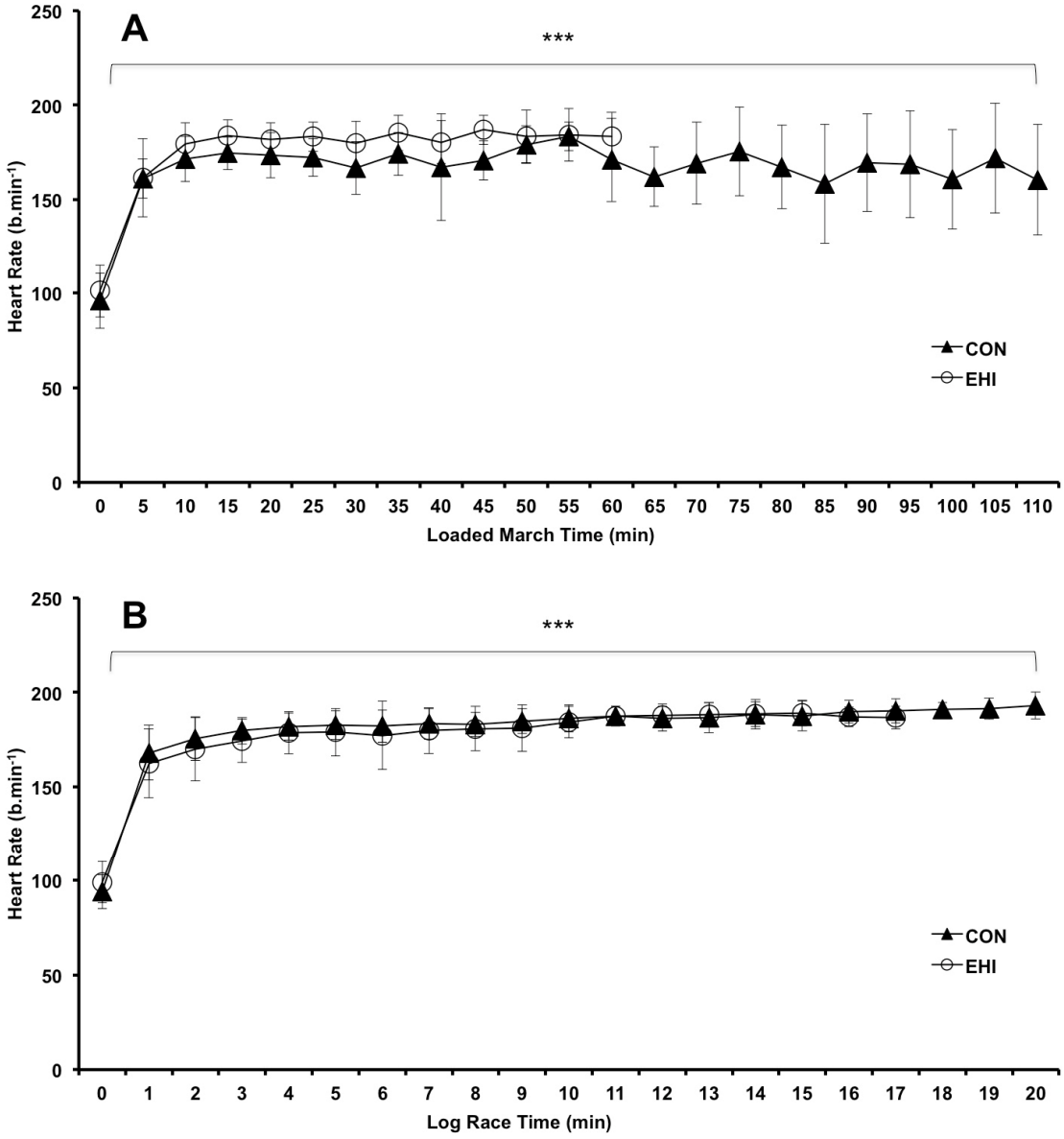


Figure 4.4: Mean (SD) heart rate (b.min-1). **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).

*** significantly different from respective pre values ($p < 0.001$).

4.3.4 Plasma Antioxidant Capacity

There was a significant main effect of time on plasma antioxidant capacity $F(1,42)= 73.9$, $p<0.001$ which were significantly elevated from pre to post LM event for LM-EHI (673.9±203.4 uM), LR-EHI (587.4±97.4 uM) and CON (587.2±80.1 uM) however no significant interaction between group was detected $F(2, 42)= 0.696$, $p=0.504$ **(Figure 4.5A)**.

Similarly, during LR event there was a significant main effect of time on plasma antioxidant capacity $F(1,34)= 80.6$, $p<0.001$, which were significantly elevated from pre to post for LR-EHI (643.8±118.7 uM) and CON (582.1±57.5 uM). While, there was no significant interaction between group on plasma antioxidant capacity $F(1,34)= 3.547$, $p=0.068$ **(Figure 4.5B)**.

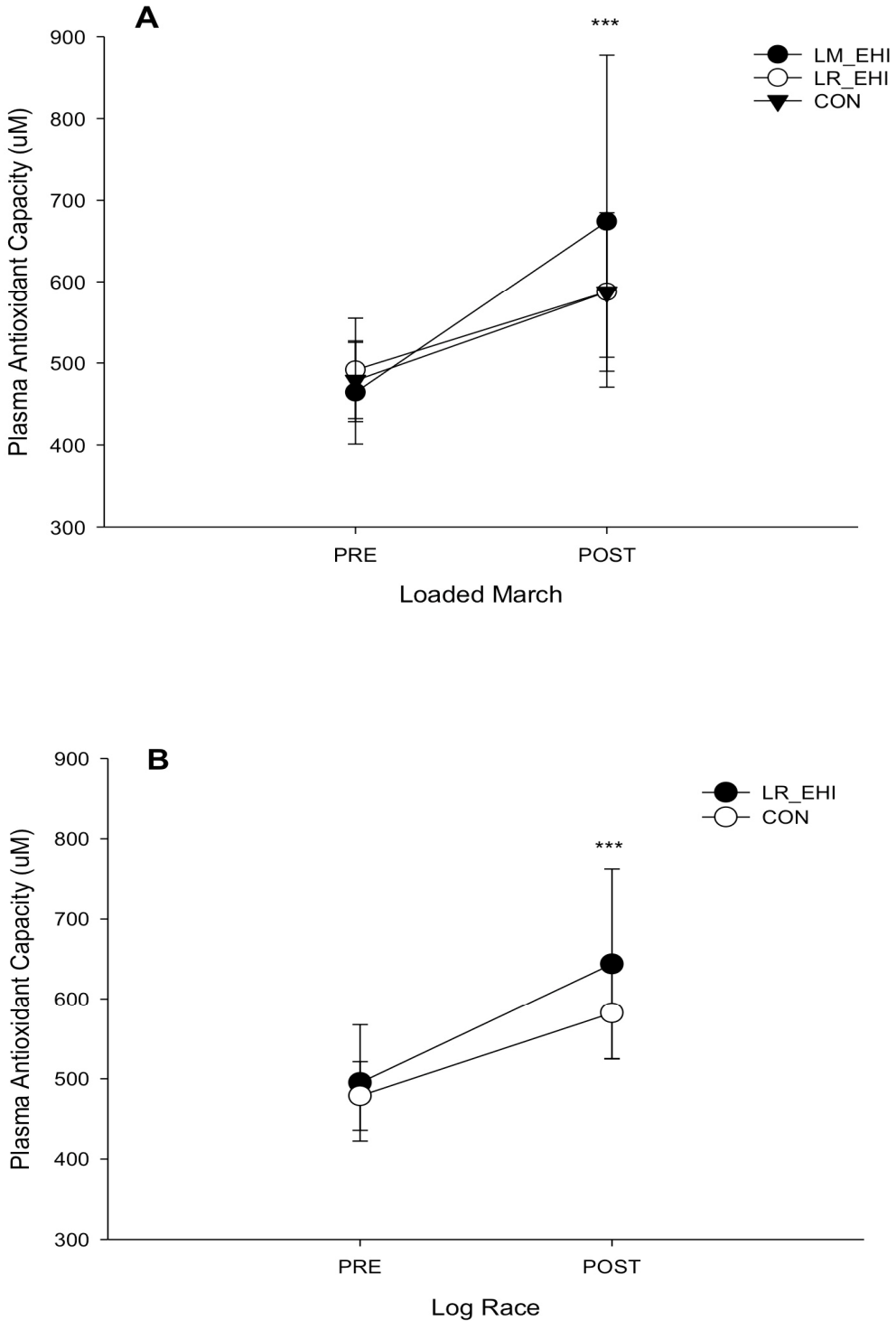


Figure 4.5: Mean (SD) plasma antioxidant capacity (μM). **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).

*** significantly different from respective pre values ($p < 0.001$)

4.3.5 Lipid Peroxidation

There was no significant effect of time on lipid peroxides from pre to post LM event for all three groups $F(1,42)= 0.182$, $p=0.672$ and there was no significant interaction between groups on lipid peroxides $F(2,42)= 0.171$, $p=0.843$ **(Figure 4.6A)**. The post LM event values for lipid peroxidation are: LM-EHI (19.3±6.7 uM), LR-EHI (19.9±9.1 uM) and CON (28.1±16.1 uM).

Similarly, during LR event there was no significant effect of time on lipid peroxides from pre post for both heat illness and CON groups $F(1,34)= 1.443$, $p=0.238$ and there was no significant interaction between groups $F(1,34)= 0.626$, $p=0.434$ **(Figure 4.6B)**. The post LM event values for lipid peroxidation are: LR-EHI (12.7±4.8 uM) and CON (14.1±9.4 uM).

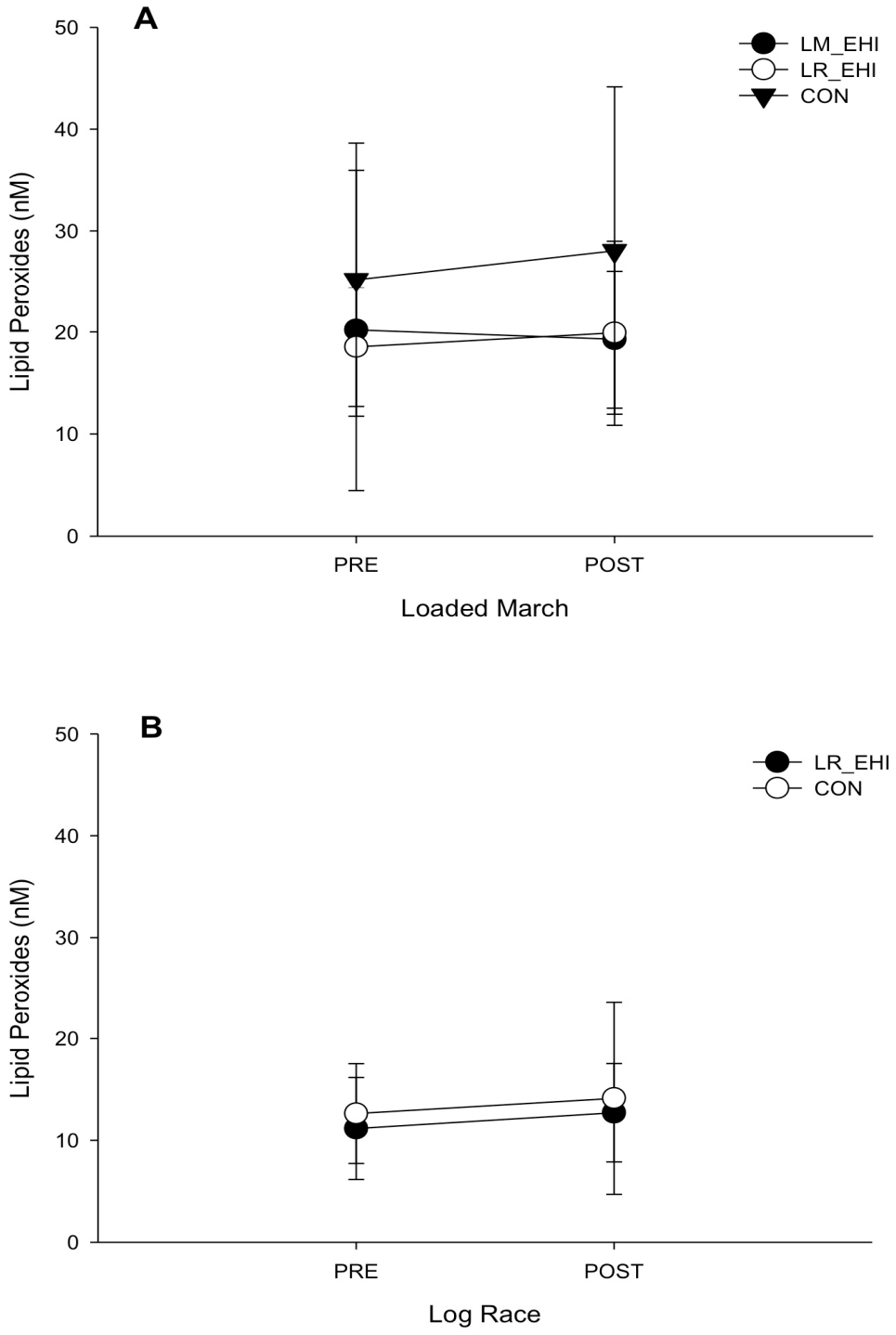


Figure 4.6: Mean (SD) lipid peroxides (nM). **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).

4.3.6 Plasma Protein Carbonyl Concentration

The between groups test indicates that there the variable group is significant $F(2,41)= 12.94$, $p<0.001$ in plasma protein carbonyl, consequently pairwise comparison revealed that LR_EHI group increased by 0.445 nmol/mg when compared to CON group ($p < 0.001$) and CON group reduced by 0.445 nmol/mg when compared to LR_EHI group ($p < 0.001$). In addition, **Figure 4.7A** showed that the lines for the two groups are rather far apart. Plasma protein carbonyl concentrations for post LM event are: LM-EHI (1.43±0.17 nmol/mg), LR-EHI (1.69±0.49 nmol/mg) and CON (1.04±0.42 nmol/mg).

However, the within subject test indicate that there was not a significant time effect, in other words, the groups do not change in plasma protein carbonyl over time $F(1,41)= 0.083$, $p=0.775$ **(Figure 4.7A)**.

From pre to post LR event, there were no changes on protein carbonyl for both heat illness and CON groups $F(1,41)= 0.083$, $p=0.775$ and there was no significant interaction between group during LR event ($p=0.424$) (**Figure 4.7B)**. Plasma protein carbonyl concentrations for post LR event are: LR-EHI (1.66±0.43 nmol/mg) and CON (1.24±0.37 nmol/mg).

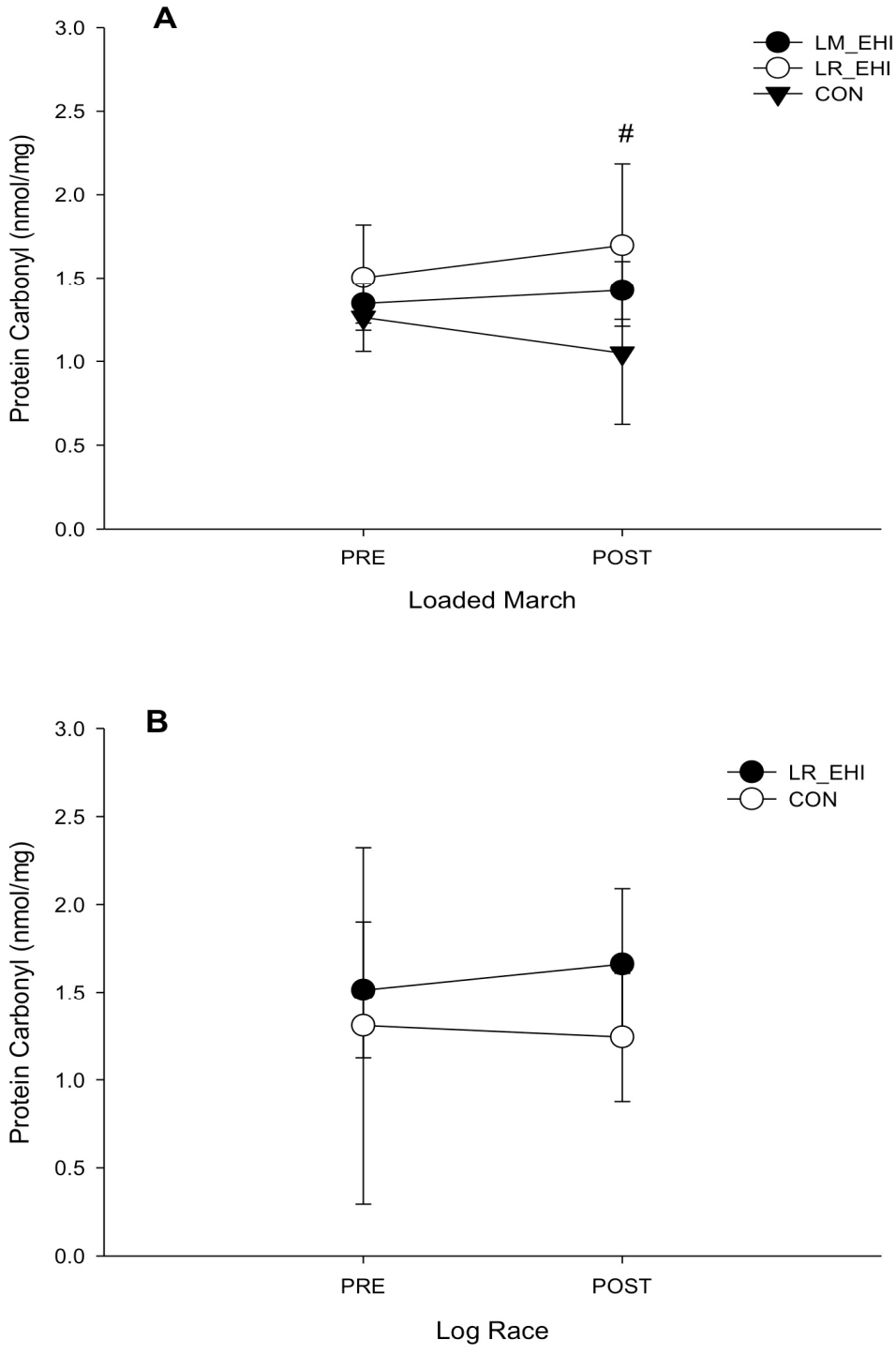


Figure 4.7: Mean (SD) plasma protein carbonyl concentration (nmol/mg). **A**) Loaded March (carried 20-kg external load ~110 min march/ran). **B**) Log Race (carried a 90-kg log ~20 running).
# denotes LR_EHI group significantly different from CON group ($p < 0.001$)

4.4 DISCUSSION

The present study investigated the redox balance after exhaustive training event, which is LM and LR in military personnel, where is EHI suspected. The present study hypothesised that the group with EHI suspected may show increasing in oxidative stress marker compared to CON group. One of the most notable finding in the present study is that the antioxidant power was significantly elevated from pre-LM to post-LM for all three groups. Similarly during LR event, plasma antioxidant capacity also increased significantly from pre to post for both CON and LR-EHI group.

In the current data set, we observed plasma antioxidant capacity was increased during both the LM and LR event. FRAP is sensitive to both aqueous and lipid phase antioxidants (Cao & Prior, 1998). This biomarker of antioxidant capacity is generally influenced in plasma by approximately 60% of uric acid concentrations (Cao & Prior, 1998). During fatigue exercise the increased in plasma uric acid may reflects elevated purine metabolism (Wayner et al., 1987). Previous study identified increases in plasma FRAP after high-intensity resistance type exercise (Hudson et al., 2008). Therefore, the increasing of antioxidant capacity for all three groups during both LM and LR event could be due to increasing of the uric acid concentration. Uric acid is believed to function as an antioxidant and known as an end product of purine metabolism (Hellsten et al., 2001). Indeed, it has been revealed that exercise to exhaustion induces a noticeable rise in plasma uric acid for a various range of exercise intensities. Kabasakalis et al. (2014) investigated the effects of swimming training modules on the redox status of adolescent swimmers and found that uric acid increased gradually after high intensity exercise.

Adenine nucleotides degradation and conversion of xanthine dehydrogenase into xanthine oxidase are suggested as the source of increasing in uric acid after exercise (Hellsten, 2000). In addition, the increased concentration of uric acid may have contributed to the antioxidant defence during recovery (Hellsten et al., 1997), as it possesses important antioxidant properties. Increased uric acid is a common finding after anaerobic (Deminice et al., 2010; Groussard et al., 2003) and aerobic exercise (Aguiló et al., 2005; González, 2008; Hellsten et al., 1997; Liu et al., 1999).

During exhaustive exercise, such as these LM and LR event, there is a greater probability that xanthine oxidase takes the responsibility for the conversion of xanthine to uric acid rather than xanthine dehydrogenase. NADH plays a role as the electron acceptor for xanthine dehydrogenase while xanthine oxidase uses molecular oxygen as the electron acceptor, generating the superoxide anion as a by-product (Hellsten, 2000), promoting oxidative stress during exercise.

The physiologic response to exercise stress may provoke increases in circulating cortisol levels by adrenal glands (Hill et al., 2008). Additionally, exercise induced releases of cortisol may directly associated with ascorbic acid efflux into the blood circulation from the adrenal glands (Gleeson et al., 1987; Padayatty et al., 2007). Hence, concomitant increases in plasma ascorbic and uric acid may reflect the enhancing of antioxidant defence system in response to extreme exercise such as LM and LR events. Thus, it may suggests that the observed increase in plasma antioxidant capacity may indicate a higher release of endogenous antioxidant into the blood stream during the LM and LR stage. However, in this present study, it is not clear that how much of the elevation in plasma antioxidant capacity is related to any specific endogenous antioxidants.

On the contrary, there were no significant changes caused by exercise on lipid peroxides for all three groups LM, LR and CON during both LM and LR event. Increased in plasma antioxidant capacity may have contributed in attenuating the plasma lipid peroxidation level. This is also in agreement with the observations by (Viguie et al., 1993) who discovered absent of changes in lipid hydroperoxides of low intensity cycle ergometer exercise at 65% V̇O2max following three consecutive days in young moderately trained individuals. Duthie et al. (1990) also found no change in plasma indices of lipid peroxidation, which is MDA after a half-marathon.

There is scarcity evidence for the occurrence of lipid peroxidation in humans during exercise and the interpretation is controversial. Several investigators have observed an increase in lipid peroxidation breakdown products (Dawson et al., 2002; Laaksonen et al., 1996; Vider et al., 2001) whereas others have reported decrease (Hubner-Wozniak et al., 1994; Rokitzki et al., 1994). Based on core body temperature data in the present study, hyperthermia was proven to increases exercise induced oxidative stress and selectively affects specific lipid oxidation markers (McAnulty et al. 2005). According to Bilzon et al., (2012) estimated levels of cardiovascular strain for LM event was equivalent to 87% and for LR event was 95% of heart rate reserve for this population of 22 year-old men which is shown that the activity was vigorous and intense, however we failed to detect any significant differences in lipid peroxidation of the plasma samples in this study.

Some data are available regarding the effects of training on lipid peroxides. Aslan et al. (1998) found that MDA level after run submaximal 15-20 min every day for 5 weeks training was lower than acute exercise. This result was consistent with Yagi (1992) who stated that blood lipid peroxide decreased in response to increased time of exercise training (to 9 months) which was implying an adaptation effect. Data also presented that;

the baseline level of LPO during LR, which was the event that occurred on Day 2, was below the baseline level during LM, which is occurred on Day 1. It could be mediated by induction of the endogenous antioxidant release upon LM event. Similar reductions in plasma lipid peroxides have been reported previously following exercise (Ginsburg et al., 1996) and this study found that susceptibility of lipids to peroxidation is reduced form pre to post exercise, therefore adding to the benefits of exercise. Therefore, absence of significantly changes in lipid peroxide in this present study for both LM and LR event may be due to our participants who are Parachute Regiment Trainees (Para) from British Army, which generally know that military duties mainly involved in endurance training that has been claimed to reduce lipid peroxidation by augmenting the body's defence capabilities. Regular training is known to decrease the accumulation of oxidative protein and DNA damage as well as heighten the resistance against ROS induced lipid peroxidation (Radak et al., 2001). This implied that antioxidant defence system with regular training might reduce the lipid peroxide level and the damage caused by free radicals.

Another finding worthy of note in the present study is protein carbonylation demonstrated a contrast result between EHI suspected group and CON group during both LM and LR event. During LM event on Day 1, the LR-EHI group responded differently in protein carbonylation compared to CON group. Similarly on Day 2, the response of protein carbonyl response was different between LR-EHI and CON group. Proteins are major targets for ROS as a result of their mainly found in biological systems (Davies, 2004; Dean et al., 1997; Stadtman & Levine, 2000). Protein carbonyl content is the most commonly used biomarker of severe oxidative protein damage (Dalle-Donne et al., 2003; Levine et al., 1990). Our hypothesis expected that the level of protein carbonyl for LM-EHI and LR-EHI would be significantly increase from pre level for the reason that the duration and intensity of both event would be sufficient to cause formation of protein carbonyl post

exercise as shown from previous studies (Bloomer et al., 2005; Bloomer et al., 2007; Goldfarb et al., 2005; Lamprecht et al., 2008) however we failed to detect any changes in protein carbonyl for the LM group (EHI suspected casualties) during the LM event. A previous study (Rahnama et al., 2007) discovered that there was no change in protein carbonyl level after exercise to exhaustion, due to aerobic training for 8 weeks. No change of protein carbonyl in LM-EHI group could be due to the equilibrium between the formation of new protein carbonyl group during the event and removal of protein carbonyl, which is present at baseline level (Wadley et al., 2016).

The increase in protein carbonyl in the LR-EHI group compared to CON during LM event could result in signalling processes leading to the induction of the heat shock proteins (HSPs) expression (Calabrese et al., 2003; Calabrese et al., 2001; Freeman et al., 1995) in order to protect cells from damage engendered by a variety of stressors such as hyperthermia (Kregel, 2002). While, reduction of protein carbonylation in CON group during both events could be some individuals exhibit unexpected responses after acute session of exercise similar to previous study (Margaritelis et al., 2014) which discovered 13% of the participants showed a decrease in protein carbonyls. This finding also consistent with some of the studies showing that protein carbonyl content were decreased after physical exercise (Chevion et al., 2003; Miyazaki et al., 2001; Shi et al., 2007) Therefore, our results opposed to the common belief, LM and LR event might not induce protein oxidative damage to the CON group.

The decreased in the protein carbonyl level in CON group during LM and LR might be as due to stimulation of a mechanism that eliminates the oxidized proteins from the blood circulation, or it could be due to the stimulation of an antioxidant mechanism that eradicates the ROS and thus prevents formation of protein carbonyl as described there

were significant increase in antioxidant power from pre to post for both event in CON group.

On top of that, review by Wadley et al. (2016) enlightened the possible mechanism which is the 20S proteasome system that could be possibly facilitating the clearance of protein carbonyl group post exercise alongside with the excretion through urine and protein uptake by the active muscle during exercise. Theoretically, all these mechanisms might be functioning actively in CON group therefore; their protein carbonyl removal might surpass the production of new protein carbonyl during exercise.

In conclusion, the results of this exploratory study showed that a noticeable increase in antioxidant power for both EHI casualties and CON during LM and LR event with no changes in lipid peroxides. While, protein carbonyl increase in EHI casualties group, but decrease in CON for both events. We suggest that the absence of lipid peroxidation in EHI is because all of our participants involved in endurance training, which is contributed in attenuating production of free radicals due to adaptation of defence capabilities. We suggested that EHI is associated with hyperthermia and hypothetically, hyperthermia is believed to increased oxidative stress, therefore considering oxidative stress as a confirmatory of heat illness use remains unclear, but its worthy of further investigation.

CHAPTER 5

Study 2: The Effects of Acute Quercetin Supplementation on Markers of Redox Balance and Extracellular Heat Shock Proteins (eHSPs) During Exercise Heat Stress.

5.1 INTRODUCTION

Exercise in hot environment may alter redox homeostasis (Flanagan et al., 1998; Laitano et al., 2010; Quindry et al., 2013). Oxidative stress is a result of perturbation between oxidant and antioxidant balance. The degree of this oxidative stress in relation to exercise has been shown to be intensity and duration dependent (Goto et al., 2003; Knez et al., 2007). It seems that the oxidative stress may be exacerbated further if exercise is undertaken in extreme environmental conditions such as at high temperatures (Laitano et al., 2010). Heat stress stimulates the production of reactive oxygen species (ROS) that induce damage to the DNA, proteins, lipids and other biological molecules lead to oxidative stress (Belhadj Slimen et al., 2014; Bruskov et al., 2002; Grasso et al., 2003).

Heat shock proteins (HSP) are a family of proteins that are produced in response to physiologically stressful conditions. Oxidative stress seems to be a stimulus for intracellular and extracellular heat shock protein production (Dimauro et al., 2016; Fittipaldi et al., 2014; Marini et al., 1996; Wallen et al., 1997; Whitham et al., 2007). These proteins play critical parts in protecting the cell from stressful conditions in order to maintain cellular homeostasis (Ghazanfarp & Talebi, 2013a; Lancaster & Febbraio, 2007). Exercise can induce production of extracellular Hsp72 (Febbraio et al., 2002; Lancaster & Febbraio, 2005a; Walsh et al., 2001) and the induction of extracellular Hsp72 by exercise is duration and intensity dependent (Fehrenbach et al., 2005). The concentration of extracellular Hsp72 also found to be highly correlated with the elevation of core temperature and ambient temperature (Gibson et al., 2014; Périard et al., 2012; Pilch et al., 2014).

It is interesting to note that oxidative stress induces HSP production (Kalmar & Greensmith 2009; Fittipaldi et al. 2014) but antioxidant supplementation may interfere with this adaptation (Fischer et al., 2006; Khassaf et al., 2003). Kuennen et al. (2011) reported that quercetin might likely to compromise heat tolerance and acclimation, thus potentially increase susceptibility to heat injury. However, despite the fact that quercetin is a potent HSP inhibitor (Hansen et al., 1997; Hosokawa et al., 1990; Hosokawa et al., 1992; Jakubowicz-gil et al., 2002; Nagai et al., 1995; Wang et al., 2009), quercetin is recognised to be a powerful scavenger of ROS (Cushnie & Lamb, 2005; Hanasaki et al., 1994) and reactive nitrogen species (RNS) (Haenen et al., 1997; Heijnen et al., 2001). In addition, quercetin is identified to possess strong anti-inflammatory, anti-carcinogenic, anti-viral, neuroprotective psychostimulant and cardioprotective capabilities (Alexander, 2006; Davis et al., 2009; Harwood et al., 2007; Oršolić et al., 2004; Read, 1995; Utesch et al., 2008). Cumulatively, these actions propose that ingestion of quercetin during exercise heat stress may reduce oxidative stress and which could potentially reduce the response of heat shock protein. However, it is not known whether quercetin supplementation could interfere with the normal adaptive response to exercise.

Two notable studies have investigated the effects of chronic quercetin supplementation to minimise exercise-induced oxidative damage. However, these studies failed to prove that chronic quercetin supplementation could counter exercise-induced oxidative stress and inflammation (Nieman et al. 2007; McAnulty et al. 2008). Given the short half-life (3.5 hours) of quercetin (Manach & Donovan, 2004), the peak plasma quercetin may have occurred much earlier than desired when quercetin was ingested 10-24 hours before the exercise. However, a previous study by McAnulty et al. (2013), investigated six days supplementation (chronic) accompanied with ingestion prior to (acute) exercise, and showed that quercetin supplementation reduced exercise-induced oxidative stress (lipid

peroxidation). The short half-life of quercetin implies that acute ingestion before or during exercise may give positive outcomes in combating exercise-induced oxidative stress leading to the hypothesis that ingesting the quercetin earlier (allow it to be fully absorbed before exercise begins) may result in a greater effect. In addition, acute antioxidant supplementation could be more efficient because chronic supplementation may hamper an adaptive response among other (endogenous) antioxidant defences (e.g., adaptation to chronic exercise training) (Chen et al., 2014; Davison & Gleeson, 2007). Simultaneous consumption of quercetin with vitamin C, folic acid and additional flavonoids increases quercetin bioavailability (Harwood et al., 2007; Konrad et al., 2011; McAnulty et al., 2013; Moon & Morris, 2007; Williamson & Manach, 2005) (Konrad et al., 2011; McAnulty et al., 2013). Antioxidants convert into oxidised forms when neutralising ROS. Quercetin is oxidised into an oquinone/ quinonmethide, known as QQ (see **Figure 2.9**) (Boots et al., 2003) when acting as a free radical scavenger. QQ can be recycled back to its parent compound with other antioxidants (ascorbate, glutathione (GSH) and NADH) and quercetin becomes available again to act as an antioxidant (Askari et al., 2012; Boots et al., 2003). An adequate plasma ascorbate level therefore should be maintained when high doses of quercetin are supplemented.

Studies examining the effect of acute quercetin supplementation during exercise in the heat are warranted, as there is a scarcity of data in this area. Hence, the aims of the present study was to examine the effects of acute quercetin supplementation undertaken 14 hours and 2 hours prior to exercise as well as during exercise on oxidative stress and heat shock response. In the present study, we examined the physiological response to exercise, thermotolerance, heat shock response as represent by plasma Hsps (eHSP70 and eHSP90α) and protein carbonyls during exercise heat stress. It was hypothesised that

acute quercetin supplementation would minimise exercise-induced oxidative stress and reduce the heat shock response during exercise in the heat.

5.2 METHODS

5.2.1 Participants

Ten male volunteers from the students of University of Bath took part in this study. Their physical characteristics and physiological capacities of the participants were presented in **Table 5.1**.

Based on previous study which was carried out by (McAnulty et al., 2004) revealed that a total 8 participants are required to achieve 80% power to detect the effect of supplementation on plasma antioxidant capacity by using one-tailed t-test for two dependent means (matched pairs) with an alpha level of 0.05. Therefore a total of 10 will be recruited to account for an anticipated 20% drop-out associated with similar previous investigations.

Table 5.1 Physical characteristics and physiological capacities of the participants (Mean ± SD).

Parameters	**Means ± SD**
Age (years)	21 ± 2
Height (cm)	1.76 ± 0.05
Body mass (kg)	71.5 ± 3.3
Maximum oxygen uptake (ml.kg^{-1} min^{-1})	54.9 ± 8.4
Body mass index (BMI) (kg.m^{2})	23 ± 1.5
Running speed at 50% $\dot{V}O_2$max (km.h^{-1})	8.0 ± 0.8
Running speed at 70% $\dot{V}O_2$max (km.h^{-1})	10.9 ± 0.9

5.2.2 Procedures

Participants were requested to come to the laboratory on five occasions. The first two visits were for preliminary tests, which included a maximum oxygen uptake ($\dot{V}O_2max$) test, familiarisation test and the remaining three visits were the experimental trials, and conducted in a randomised order.

5.2.3 Preliminary tests

For maximum oxygen uptake ($\dot{V}O_2max$), participants were required to perform a continuous incremental running protocol until exhaustion on a motorised treadmill (Woodway ELG70, Weiss, Germany). The test protocol was modified from Taylor et al. (1955). Familiarisation trials were carried out as similar as the main experimental trial which all the participants ran in the heat at 70% $\dot{V}O_2max$ for 60 min

Approximately two weeks after familiarisation trials, participants visited the lab for their first experimental trial between 08:30 and 9:00 following an overnight fast from 23:00. However, participants were permitted to drink plain water and were requested to consume 500 mL of plain water one to two hours before exercise. Participants were asked to abstain from alcohol; caffeine and refrain from strenuous exercise the preceding 48 hours.

5.2.4 Main experimental trials

The experimental protocol is shown in **Figure 5.1** and the protocol details are described as follows. All participants were randomly assigned, within crossover design, to complete three trials: Quercetin (Q), Quercetin plus vitamin C (QC), or placebo (P). There were at least 14 days between trials. Participants were required to run for 60 min at 70% of their $\dot{V}O_2max$ or until rectal temperature (Trec) reached 39.5°C. All exercise trials P, Q and QC took place in regulated environmental chamber; room temperature and relative humidity were well maintained as shown in **Table 5.2.** Absence of significant different in exercise

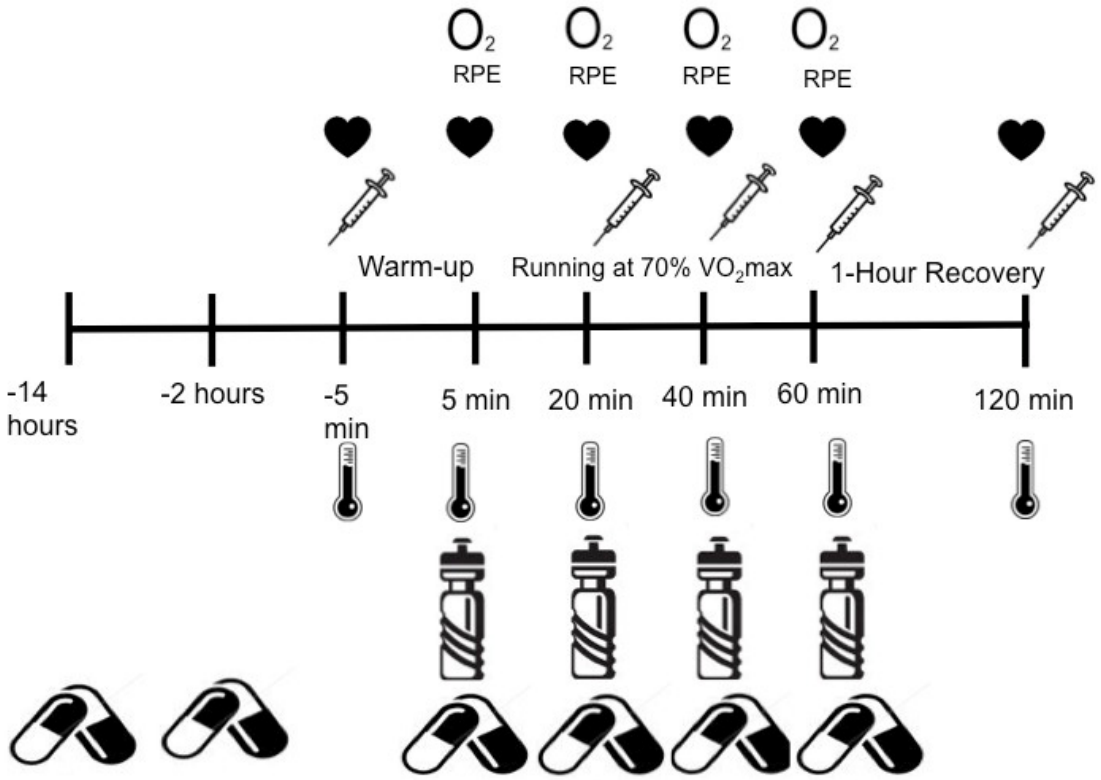
intensity (**Table 5.2**) between trials may indicate that the intensity was well controlled and supplementation did not reduce the exercise strain.

Table 5.2 Environmental conditions and exercise intensity for all trials (Mean ± SD).

	Placebo	**Q**	**QC**	***P*-value between trials**
Room Temperature (°C)	32.7 ± 0.3	33.1 ± 0.3	33.3 ± 0.4	*p = 0.110*
Relative Humidity (%)	26.5 ± 2.9	28.3 ± 3.4	29.9 ± 2.5	*p = 0.095*
Intensity (70% $\dot{V}O_2$max)	69.3 ± 0.15	68.2 ± 0.9	70.3 ± 1.5	*p = 0.987*

Participants were randomly assigned by an electronic research randomisation generator (http://www.randomization.com) to consume either tablets of 1000mg Quercetin (Q), 1000mg Quercetin + 1000mg Vitamin C (QC) or placebo (P) 14 hours before (7:30pm the night before) and 2 hours before (7:30am) exercise, participants were consumed tablets of 500 mg Quercetin, 500mg Quercetin + 500mg Vitamin C or placebo (Davison & Gleeson, 2007).

Participants also consumed 3 $mL.kg^{-1}$ body weight of plain water with a tablet of either 500 mg Quercetin (Q), 500mg Quercetin + 500mg Vitamin C (QC) or placebo (P) every 20 minutes during running. Heart rate, rectal temperature, thermal discomfort scale and ratings of perceived exertion (RPE) were recorded pre-warm up, at the end of warm up, every 10 minutes throughout the exercise trial, at the end of exercise and post-one hour of exercise trial. Expired air samples were collected pre-warm up, at the end of warm up, every 20 minutes throughout the exercise trial and at the end of exercise trial. Blood samples were obtained pre warm-up, at intervals of 20 minutes during the trial, at the end of exercise trial and post one-hour exercise trial.



RPE: Rating of perceived exertion

O_2 : Expired air collection

: Heart rate

: Blood sampling

: Rectal temperature

: Plain water ingestion

: Antioxidant supplementation

Figure 5.1 Study experimental protocol

5.2.5 Blood samples

Blood samples were drawn from antecubital vein of the participant into the EDTA collection tube. Heamoglobin and haematocrit concentrations were analysed by using an automated haematological analyser (Sysmex KX-21N). Subsequently, plasma volume was determined (Dill & Costil, 1974).

Following centrifugation, plasma was separated and stored in 1.5mL aliquots at -80°C for before analysis. Samples were analysed subsequently for concentration of protein carbonyls (corrected for total plasma protein), plasma heat shock protein 70 (eHSP70), plasma heat shock protein 90 (eHSP90α), ferric reducing ability of plasma (FRAP) and plasma quercetin. Given that changes in plasma volume can affect blood concentrations of biochemical markers (Kargotich, Goodman, Keast, & Morton, 1998), all measurements were corrected for plasma volume changes.

5.2.5.1 Protein Carbonyls

Protein carbonyl is a measure of protein oxidation; it was measured by enzyme-linked immunosorbent-assay (ELISA) according to the procedures recommended by the manufacturer (Oxiselect, Cell Biolabs, San Diego, USA) as described in the general methods (**Chapter 3, section 3.9.2**).

5.2.5.2 Plasma Heat Shock Protein 70 (eHSP70)

Circulating eHSP70 was analysed in duplicate by a commercially available ELISA kit which is ENZ-KIT-101-001 Amp'd® Hsp70 high sensitivity ELISA kit ELISA kit (Enzo Lifesciences, Lausen, Switzerland) as described in the general methods (**Chapter 3, section 3.9.4**).

5.2.5.3 Plasma Heat Shock Protein 90 (eHSP90α)

Circulating eHSP90α was assessed in duplicate using ELISA kit, which is specified for the detection of human HSP90α (AQDI-EKS-895, Enzo Lifesciences, Lausen, Switzerland) as described in the general methods (**Chapter 3, section 3.9.5**).

5.2.5.4 Plasma Antioxidant Capacity (FRAP)

Plasma antioxidant capacity was assessed in plasma using the ferric reducing ability of plasma (FRAP) assay established by (Benzie & Strain, 1996) as described in the general methods (**Chapter 3, section 3.9.3**).

5.2.5.5 Plasma Quercetin

Total plasma quercetin (quercetin and its primary metabolites) was measured using liquid chromatography–tandem mass spectrometry as previously described (Wang & Morris 2005) as described in the general methods (**Chapter 3, section 3.9.6**).

5.3 Statistical analysis

All statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS version 24). Two-way ANOVA with repeated measures was used to analyse all variables. Where sphericity was broken, P values were corrected for by using the Greenhouse-Geisser method. All the statistical significance was accepted at $p < 0.05$. All data were expressed as means ± standard deviation (SD).

5.4 RESULTS

5.4.1 Heart rate

The present study showed that heart rate for all trials were significantly increased throughout the exercise trials (*p<0.001*) (**Figure 5.2**). However, there was no significant interaction between all trials, F(5.2,60.4)=0.512; *p=0.775*.

Mean heart rate at the end of the P, Q and QC trials in the present study were 184.8 ± 9.9 beats.min-1 (92.9 ± 1.2% of HRmax), 185.7 ± 8.9 beats.min-1 (93.3 ± 1.2% of HRmax) and 185.0 ± 7.0 beats.min-1 (93.1 ± 1.2% HRmax) respectively. There were no statistically significant differences between P, Q and QC trials showing that all participants underwent a similar cardiovascular strain. One-hour post exercise trial, heart rate of the participants for P, Q and QC trials were returned nearly to baseline level 72.3±9.5, 69.1±12.9 and 73.3±19.0 beats.min $^{-1}$ respectively.

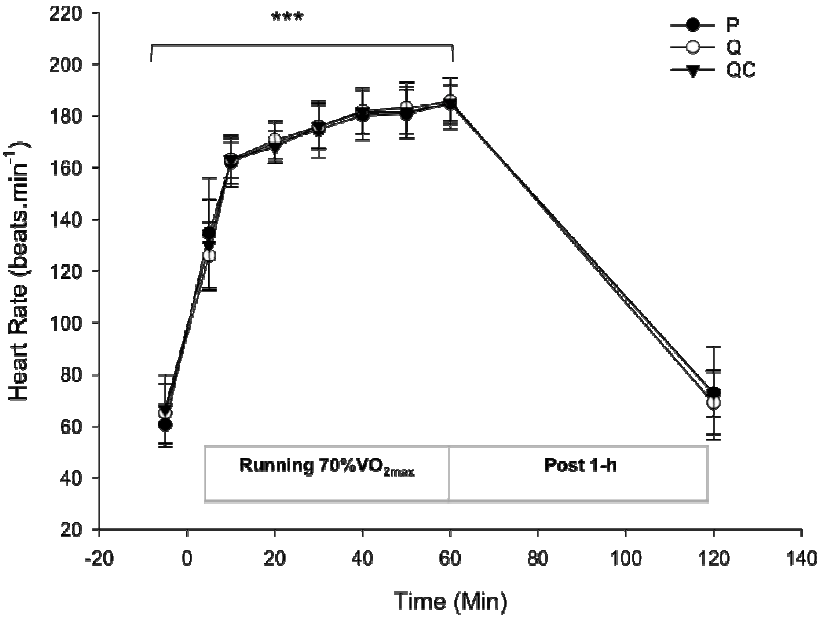


Figure 5.2 Heart rate (beats.min^{-1}) in Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise for all trials p<0.001

5.4.2 Rectal temperature

Throughout exercise, rectal temperature (T_{rec}) increased significantly during each trial (*p<0.001*) (**Figure 5.3**) and there was no significant interaction between trials F(6.0,60.1)=0.592, *p=0.736* at each time point. T_{rec} at the end of exercise for P, Q and QC trials was 39.10 ± 0.3 1°C, 39.20 ± 0.25 °C and 39.30 ± 0.25 °C respectively. One-hour post exercise trials, T_{rec} of the participants for P, Q and QC trials were decreased to 36.98 ± 0.21°C, 36.98 ± 0.12 °C and 36.94 ± 0.25°C respectively.

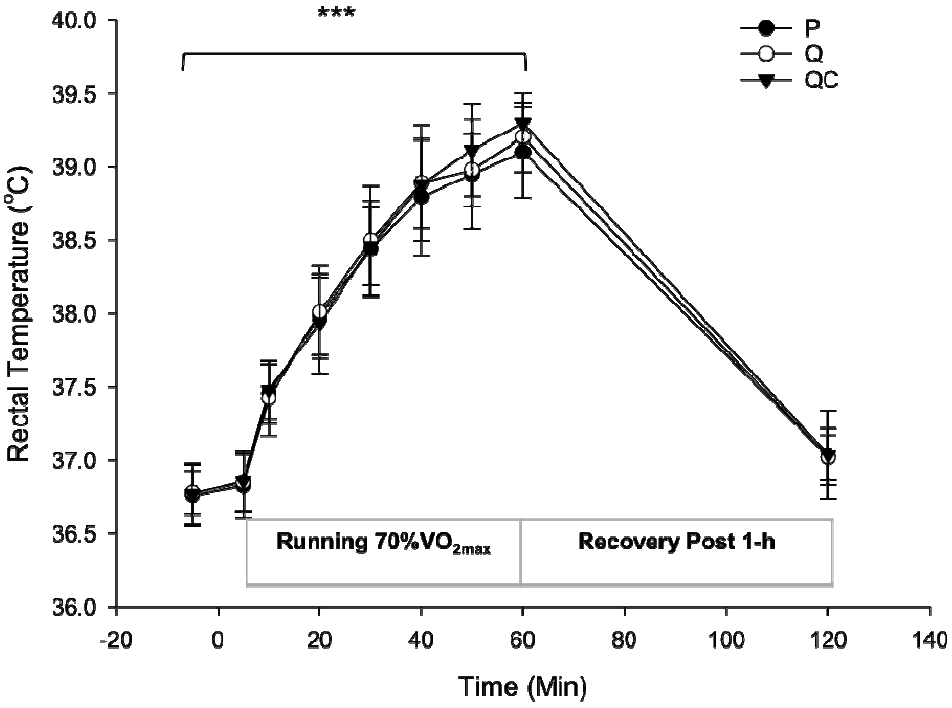


Figure 5.3 Rectal temperature (ºC) in Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise level for all trials p<0.001

5.4.3 Physiological Strain Index (PSI)

PSI significantly increased over time for all trials (F(1.165, 28.0)=103.0, *p<0.001*) (**Figure 5.4**). The percentage of PSI for P, Q and QC trials were increased by 45.4%, 42.9% and 40.7% respectively when compared to pre exercise. However, no significant interaction was found in any of the supplementation regimens F(2.3,28)=0.790, *p=0.481*.

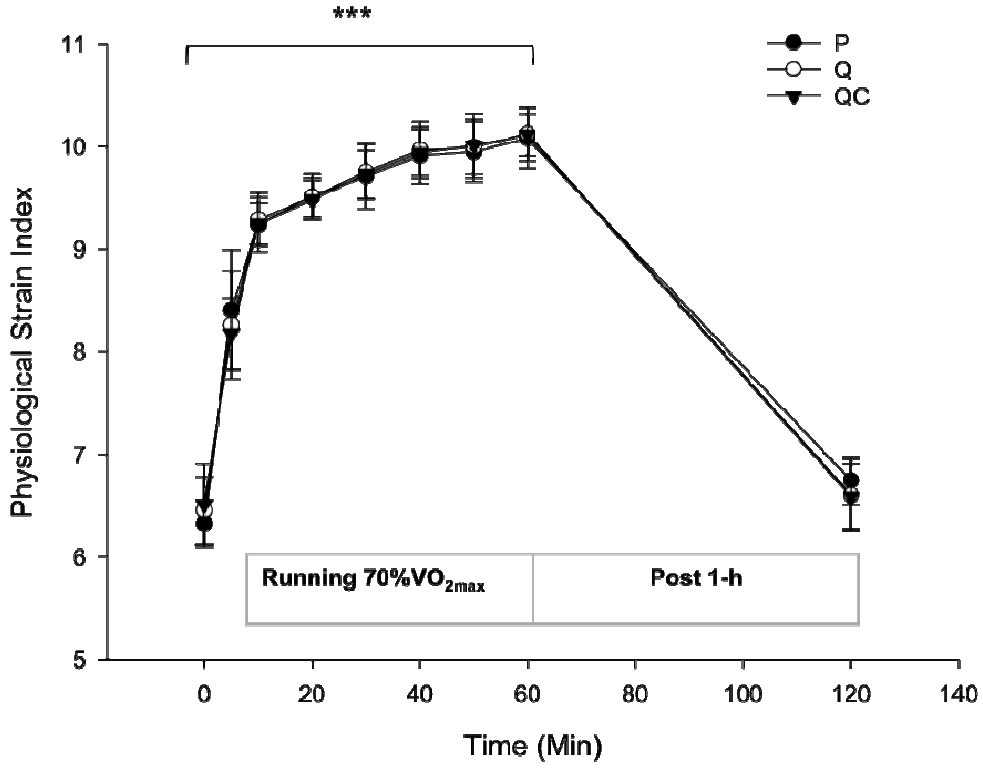


Figure 5.4 Physiological Strain Index (PSI) in Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise level for all trials p<0.001

5.4.4 Ratings of Perceived Exertion (RPE)

There was a progressive increase in RPE during exercise reaching near maximum ratings at the end of exercise ($F(1.8,39.9)=186.8$, *p<0.001*) (**Figure 5.5**). There were no differences in RPE between any of the supplementation trials, $F(2, 22)=0.756$, *p=0.481*.

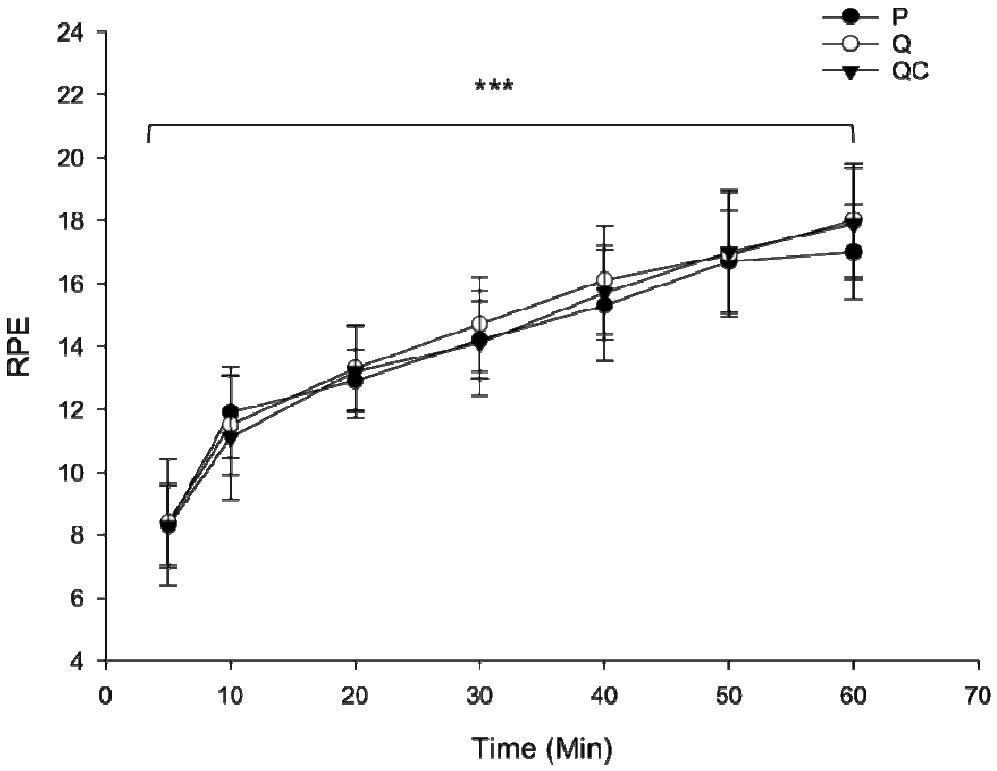


Figure 5.5 Rating of Perceived Exertion (RPE) in Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise level for all trials p<0.001

5.4.5 Haematological parameters

5.4.5.1 Plasma Quercetin

No significant interaction F(2,27)=1.97, *p=0.16* exists between the P, Q and QC trials at any time point however there were significant differences over time F(2.5,68.35)=3.68, *p=0.02* (**Figure 5.6**). Post hoc tests using the Bonferroni correction revealed that plasma quercetin only increased significantly for QC trials by 500ug.L^{-1} from pre to post one hour of exercise trial. In contrast, there were no significant differences at each time point when compared to pre exercise for P and Q trials. Mean plasma quercetin at rest for P, Q and QC trial was 535.3 ± 428.6.0ug.L^{-1}, 639.7 ± 491.7.0ug.L^{-1} and 867.1 ± 675.6ug.L^{-1} respectively, it showed that before exercise started the level of quercetin in QC trial was slightly higher compare to P regardless of interaction effect.

Mean plasma quercetin throughout the exercise trials for P, Q and QC was 541.1 ± 360.0 ug.L^{-1}, 845.6 ± 653.0 ug.L^{-1} and 1117.1 ± 843.1 ug.L^{-1} respectively. Even though there was not a significant interaction effect, the mean plasma quercetin level throughout the exercise for QC trial was double the mean plasma quercetin level for P trial. In addition, post one-hour exercise; the plasma quercetin levels was decreased about 12% for P trial, while for the Q and QC trials the plasma quercetin levels were increased by 61% and 58% above pre exercise levels in the Q and QC trials, respectively.

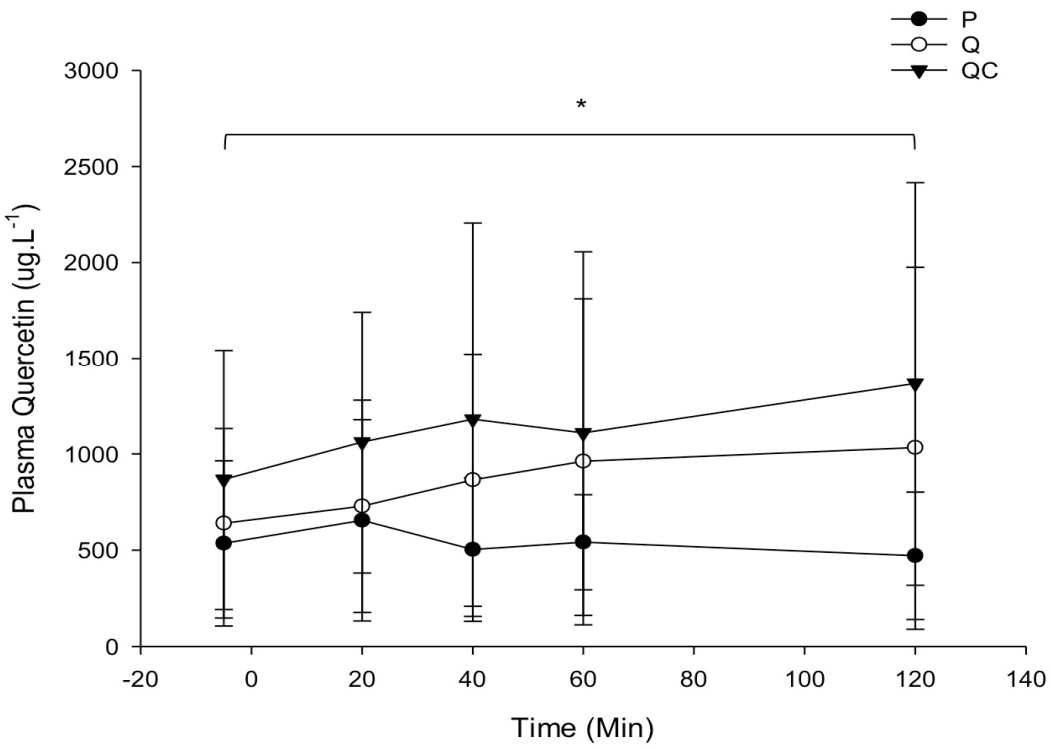


Figure 5.6 represents plasma quercetin (ug/L) for each exercise trial for each exercise trial Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

* denotes significantly difference from pre exercise level for Q and QC trials $p<0.05$

5.4.5.2 Plasma Heat Shock Protein 70 (eHSP70)

The level of eHSP70 was significantly increased compared to pre level of eHSP70 for all trials F(1.4,54)=6.31, *p=0.009* (**Figure 5.7**).

Post hoc tests using the Bonferroni correction revealed that exercise heat stress elicited a slight increase in eHSP70 for P, Q and QC trials by 0.32 ng.mL^{-1}, 0.31 ng.mL^{-1} and 0.69 ng.mL^{-1} respectively between pre and at the end of exercise trials. In addition, when compared post one-hour with pre exercise, there was also a slight increase in eHSP70 for P, Q and QC trials by 0.35 ng.mL^{-1}, 0.35 ng.mL^{-1} and 0.39 ng.mL^{-1} respectively.

However no significant interaction F(2,27)=0.72, *p=0.931* exists between the P, Q and QC trials at any time point.

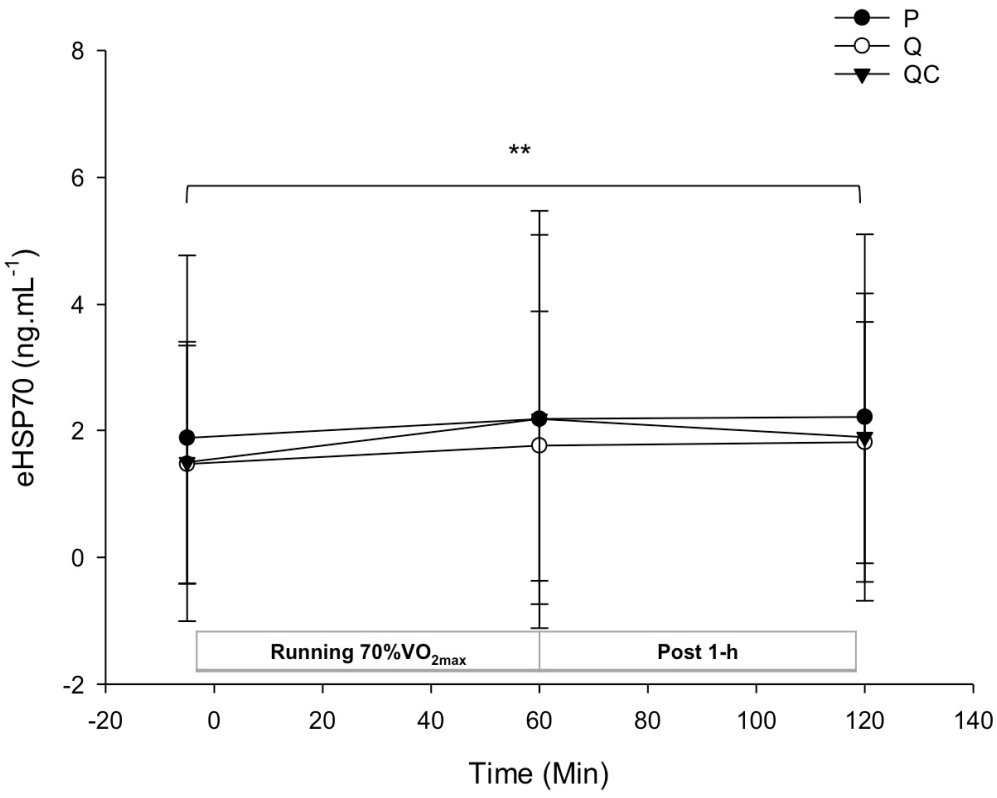


Figure 5.7 represents eHSP70 (ng.mL^{-1}) for each exercise trial Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

** denotes significantly difference from pre exercise level p<0.01

5.4.5.3 Plasma Heat Shock Protein (eHSP90α)

There was significant differences over time F(2,54)=27.43, *p<0.001*, eHSP90α was significantly increased compared to pre level of eHSP90α (**Figure 5.8**).

Post hoc tests using the Bonferroni correction revealed that from pre to post exercise, the heat stress elicited a slight increase in eHSP90α for P, Q and QC trials by 13.5 ng.mL^{-1}, 9.0 ng.mL^{-1} and 10.8 ng.mL^{-1} respectively. In addition, from pre to post one-hour exercise, there was also a slight increase in eHSP70 for P, Q and QC trials by 10.7 ng.mL^{-1}, 13.5 ng.mL^{-1} and 10.9 ng.mL^{-1} respectively.

However no significant interaction F(2,27)=0.33, *p=0.967* exists between the P, Q and QC trials at any time point.

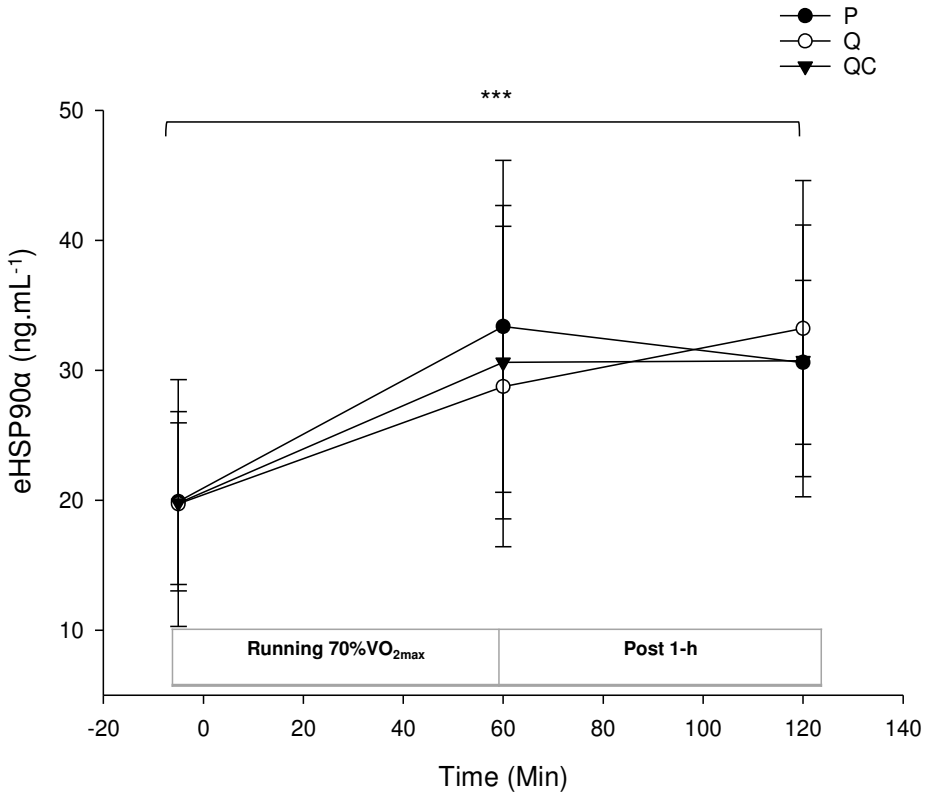


Figure 5.8 represents eHSP90α (ng.mL^{-1}) for each exercise trial Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise level p<0.001

5.4.5.4 Protein Carbonyl

There were no significant differences over time F(2,54)=1.094, *p=0.327* and no significant interaction effects F(2,27)=6.20, *p=0.545* (**Figure 5.9**).

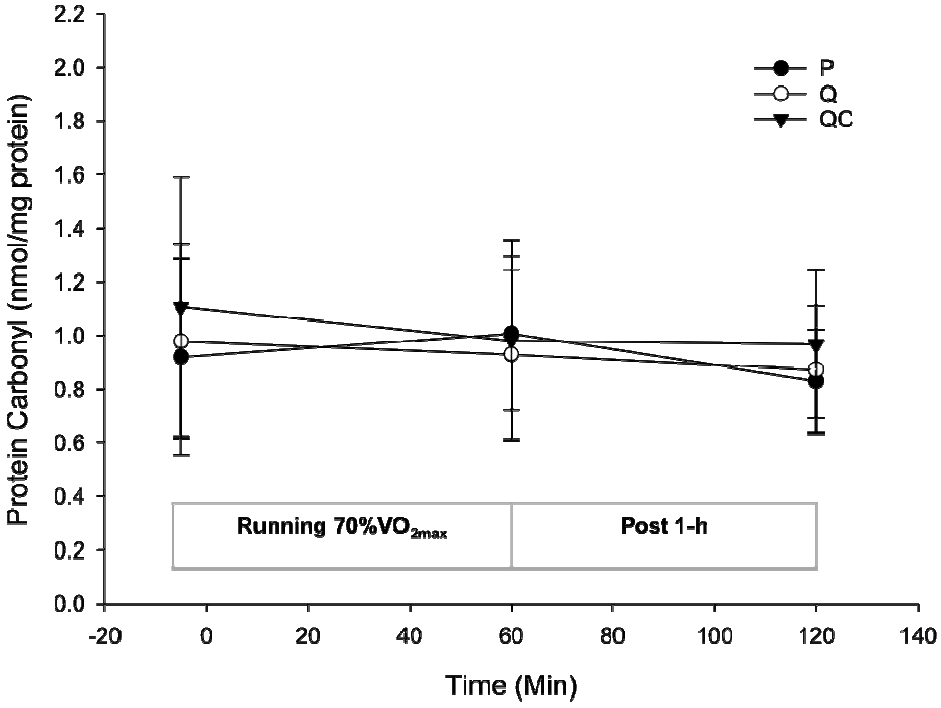


Figure 5.9 represents plasma protein carbonyl concentration (nmol/mg protein) for each exercise trial Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

5.4.5.5 Plasma Antioxidant Capacity (FRAP)

There were significant differences over time F(2,54)=25.55, *p<0.001*, plasma antioxidant capacity was significantly increased compared to pre level (**Figure 5.10**). Post hoc tests using the Bonferroni correction revealed that plasma antioxidant capacity increased for P, Q and QC trials by 71.3uM, 113.4uM and 99.4uM respectively, between pre and post one hour of exercise trials but no significant differences between pre and at the end of exercise.

However there was not a statistically significant interaction effect between the P, Q and QC trials F(2,27)=0.14, *p=0.953*.

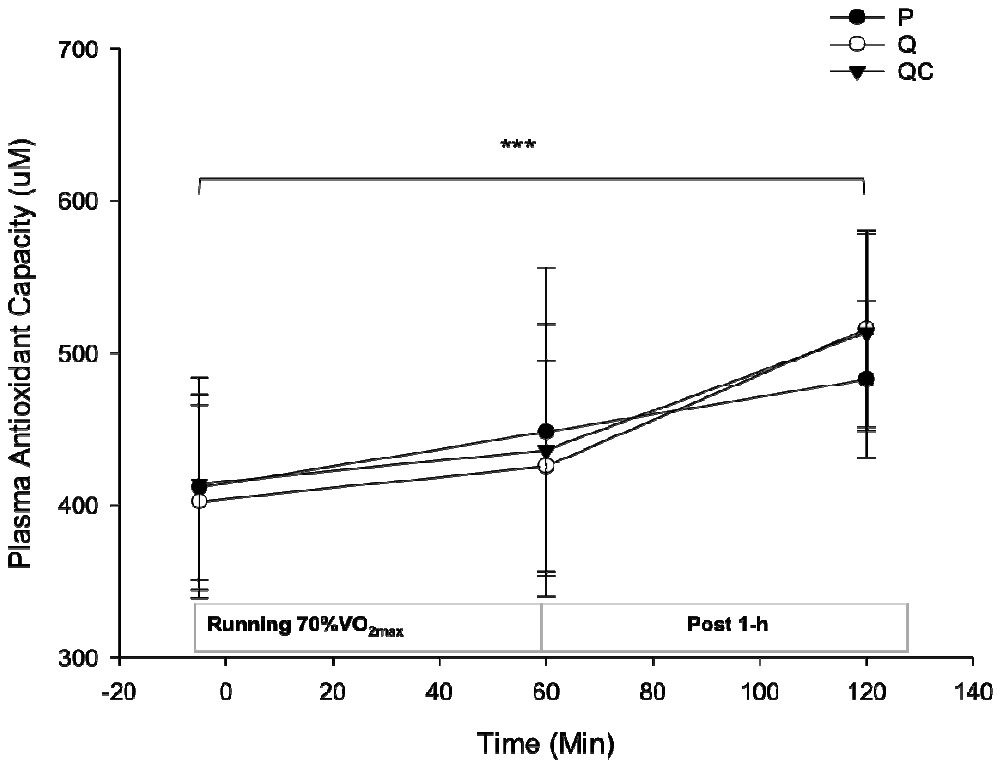


Figure 5.10 represents plasma antioxidant capacity (FRAP) (uM) for each exercise trial Placebo (P), Quercetin (Q) and Quercetin plus Vitamin C (QC) trials (Mean ± SD).

*** denotes significantly difference from pre exercise level p<0.001

5.5 DISCUSSION

The purpose of this study was to investigate the effects of acute quercetin supplementation with 14 hours and 2 hours prior to exercise as well as during exercise heat stress on markers of redox balance and extracellular heat shock protein (eHSPs) during exercise heat stress. To the best of our knowledge, this is the first study to assess the acute effects of antioxidant supplementation during exercise heat stress. The present study demonstrated that eHSP70, eHSP90α and antioxidant power (FRAP) increased significantly as a result of exercise heat stress however, this response was not affected by supplementation. In addition, exercise heat stress failed to induce changes in plasma protein carbonyl concentration, a biomarker of oxidative stress.

Extracellular HSPs (eHSPs) are cellular messengers that respond to stress, injury, infection and cell damage. Increased levels of eHSP70 in all trials were observed during exercise and this could be due to the exposure of the heat in combination with exercise thus leading to hyperthermia. Gibson et al. (2014) showed that the levels of plasma HSP72 increased about 21% post cycling at 50% $\dot{V}O_2$ peak in hot environment (30.2°C), similar to the present study demonstrated that eHSP70 increased about 29% post running at 70%$\dot{V}O_2$max in hot environment (33.0°C) when compared to pre exercise level. The percentage increased in rectal temperature (7%) and peak rectal temperature at the end of exercise (39.0°C) in the present study were also similar with Gibson et al. (2014). The level of HSPs increased in the present study could be associated with the rise of core temperature (hyperthermia) as demonstrated from previous study, walking at 50% $\dot{V}O_2$max until core temperature reached 38.5°C in both conditions; high and low rates of heat storage resulted in a similar extracellular Hsp72 (eHSP72) response (Amorim et al., 2008).

In support, previous work has shown that there is a strong relationship between eHSP72 (Périard et al., 2012) and iHsp72 (Périard et al., 2015) with the level of hyperthermia after exercise at 60% and 75% $\dot{V}O_2$max in hot conditions (40°C, 50% RH). However, in contrast to eHSP72, intracellular Hsp72 (iHsp72) continued to increase during the 24 hours period after exercise in hot condition (Périard et al., 2015). It has also been reported that although eHSP72 levels corresponded to the duration of exercise heat stress and recovery however; the 15% increase in eHSP72 observed immediately after exercise returned to baseline levels 1 hour post-exercise. But, the response of iHsp72 to exercise heat stress remained elevated and high even after 24 hours recovery (~2.5-fold baseline values) (Lee et al. 2017). The finding of a significant increase in eHSP70 in the present study for all trials following exercise heat stress supports the idea that exercise and heat stress causes a release of eHSP70.

Under conditions such as exercise heat stress in the present study, eHSPs could be released from cells into the extracellular environment or enter the systemic circulation (Calderwood et al., 2007) facilitate defense to stress challenges. Researchers (Gibson et al., 2014; Ogura et al., 2008) suggested that body temperature elevation as exposed in the present study (**Figure 5.3**), increased circulating catecholamines (Iguchi et al., 2012; Martin Whitham et al., 2006), exercise response (Whitham et al. 2007), and environmental stress (Lee et al. 2015) play a role in stimulation of eHSP70.

Quercetin is known as a potent HSP inhibitor (Hosokawa et al., 1990; Hosokawa et al., 1992). The mechanism of this inhibition has been suggested to involve blocking the heat shock factor-1 (HSF-1) activation after heat stress and thus inhibit heat-induced up-regulation of HSP70 (Nobuko Hosokawa et al., 1992). Kuennen et al. (2011) revealed that

daily application of quercetin compromised the thermotolerance and heat acclimation as well as blunted the expression of HSP72 in peripheral blood mononuclear cells (PBMC) with 7 days supplementation of 2000 mg/day of quercetin. However, in the present study, acute quercetin supplementation does not blunt the response of eHSP70 during exercise heat stress. Our results are in contrast to those of others (e.g. Kuennen et al. (2011) because we measured extracellular HSP, as opposed to intracellular HSP (iHSP).

In addition, the present study also demonstrated that the acute supplementation of quercetin induce similar effects on heart rate response, rectal temperature and PSI when compared to placebo (**Figure 5.2**, **Figure 5.3** and **Figure 5.4** respectively). Similar finding as discovered in previous study (Cheuvront et al., 2009), the author found that acute supplementation of quercetin elicited similar effects on the heart rate response and rectal temperature throughout the exercise trial in the heat (40°C, 20–30% relative humidity) when compared to placebo. Thus, it might indicate that acute quercetin supplementation at least 14 hours before exercise in the heat might not affect thermoregulation during exercise in the heat.

Other than HSP70, HSP90 also have been associated with stress and exercise (Fehrenbach et al., 2000; Locke et al., 1990). Due to the anti-cancer properties of quercetin, this supplement has been examined extensively in cancer research as a flavonoid, which can induce apoptosis by down regulating the expression of HSP90 (Aalinkeel et al., 2008; Zanini et al., 2007). However, in the present study as shown in **Figure 5.8,** quercetin does not inhibit the response of eHSP90α induced by exercise heat stress. Indeed, a similar results have been shown in an animal study (Chen et al., 2014), which reported that that acute quercetin supplementation does not affect the heat shock response (HSP70, HSP90 and HSF-1) in muscle, heart and liver tissues.

Quercetin may be ineffective because of the short duration of supplementation (acute), this might not be capable to prevent the up-regulation of both eHSP70 and eHSP90α, thus indicate that acute supplementation might not affect the heat shock response however, the mechanism behind this is still unclear.

Evidence has suggested that oxidative stress is related to exercise intensity. The majority of the studies discovered that increase in oxidative stress biomarkers in aerobic activities varies between 65%$\dot{V}O_2$max to 75%$\dot{V}O_2$max with exercise duration vary from minutes to several hours (Finaud et al., 2006; Goto et al., 2003). As a result of the action of reactive oxygen species (ROS) induced by exercise, the circulating and tissue proteins are prone to be carbonylated, thus carbonyl proteins are formed (Laitano et al., 2010). However, in the present study, there were no changes discovered in protein carbonyls level (**Figure 5.9**) during exercise heat stress regardless of the supplementation trials (P, Q and QC). These results are similar to those of Souza-Silva et al. (2016) who reported no changes in protein carbonyls after high intensity interval training (HIIT) in the heat (35°C, 55% relative humidity) compared to temperate environment (22°C, 55% relative humidity).

The QC supplement was designed in the present study to improve the bioavailability and bioactive effects of quercetin. Previous research has suggested that simultaneous ingestion of quercetin with vitamin C, folate, and additional flavonoids improves the bioavailability of the quercetin (Harwood et al., 2007; Manach et al., 2005; Moon & Morris, 2007). In addition, the study design to consume the supplements the day before exercise (14 hours before), and then again 2 hours before exercise, and every 20 minutes during exercise trial are to maintain the bioavailability of the antioxidant in the blood. Therefore, based on the level of plasma quercetin in Q and QC trials (**Figure 5.6**), it indicated that the study design successfully increased and maintained the level of quercetin in the blood

during Q and QC trials if compared to P trial. However, the absence of interaction effect between trials could be due to large variation of plasma quercetin level between participants. One possible reason might be participants were not told to restrict their intake of foods rich in flavonoids content (e.g as blueberries, strawberries, apples, celery, oranges and etc.), however all the participants were asked to refrain from consuming any mineral or vitamin supplement (other than those provided), or any other antioxidant supplements for 2 weeks before and during the trials. Therefore, this would be one of the study limitations as consuming food contained of flavonoid might affects the level of plasma quercetin.

Although this present study observed elevated concentrations of quercetin in the plasma of both-supplemented groups (Q and QC), in contrast to our hypothesis, this plasma increase did not affect plasma antioxidant capacity and plasma protein carbonyl concentration. This finding is in agreement with data obtained in other human intervention trials examining the potential effects of quercetin supplementation on antioxidant biomarkers (McAnulty et al., 2013; McAnulty et al., 2008, 2011; Quindry et al., 2008) who found no alterations in oxidative damage marker with chronic intake of quercetin before exercise (between 7 days to 6 weeks). However, several studies (Chang et al., 2010; Goldfarb et al., 2005; Morillas-Ruiz et al., 2005; Morillas-Ruiz et al., 2006) revealed that antioxidant supplementation flavonoid-based successfully reduced certain biomarkers of oxidative stress (e.g lipid oxidation (TBARS), protein carbonyl and malondialdehyde (MDA) and increased ferric-reducing ability of plasma (FRAP). The contradictory results presented here to those previous studies could be due to methodological differences, antioxidant defenses adaptation in well-trained athletes, type of biomarkers used to detect oxidative damage,

various duration of supplementation, supplementation type (tablet or beverages) and source of supplementation.

Plasma antioxidant capacity increased significantly as a result of exercise heat stress, but not due to quercetin ingestion, as there was no significance interaction between trials. This increase in plasma antioxidant capacity presumably represents the release of urate and ascorbate into the blood during exercise (Aguiló et al., 2005; Yanai & Morimoto, 2004). In vitro, quercetin exhibits powerful antioxidant activity because of the presence of a 3,4 B-ring hydroxyl group configuration (Shashank & Abhay, 2013). Therefore, the plausible reason for the lack of change in plasma antioxidant capacity in vivo could be because of substitution of the 3,4 B- ring hydroxyl groups with methyl or glycosyl groups during metabolism in humans, thus abolishing a large amount of the free radical scavenging ability of quercetin detectable by the FRAP (Manach & Donovan, 2004; Manach et al., 1998).

Conversely, no changes were detected in protein carbonyl level in all trials. This undetectable changes occurred despite the increased of plasma quercetin and plasma antioxidant activity as there were no interaction effect between trials. Bloomer et al. (2007) revealed that protein carbonyls were greater following the 120-minute cycling at 70% $\dot{V}O_2$peak and this increment of protein carbonyls remained elevated for a longer time course compared to the 30 and 60-minute conditions. This study suggested that exercise duration threshold is necessary for further production of protein carbonyls (>60 minutes of continuous exercise). McAnulty et al. (2013) also supported the theory that protein carbonyl was increased after exercise for one-hour running at a 3% grade and at 80% $\dot{V}O_2$max however this increment were not affected by 7 days resveratrol and quercetin supplementation. Since the protein carbonyls were not different between the trials at any

time points (pre exercise, immediately post exercise and one-hour post exercise), implying that proteins were protected from oxidative stress-induced damage throughout the exercise heat stress. These observations indicated that the absence of changes in protein carbonyl might due to the upregulation of heat shock proteins (HSPs), as eHSPs have been found to increase in the plasma after exercise heat stress for all trials (**Figure 5.7** and **Figure 5.8**). Therefore it is reasonable to indicate that the absence of protein oxidation observed after exercise in the heat could have been a result of protection provided by heat-induced upregulation of HSPs This outcome would be consistent with previous evidence that HSPs may serve as an supplementary antioxidant (Fittipaldi et al., 2014; Oksala et al., 2014).

In addition, Wadley et al. (2016) suggested that the possible mechanism by which protein carbonyls are cleared is the 20S proteasome system that could be possibly facilitating the removal of protein carbonyl group post exercise alongside with the excretion through urine and protein uptake by the active muscle during exercise. In addition, the 26S proteasome is an integral part of the cell's mechanism to degrade proteins, Hsp90 found to be interacts with the 26S proteasome and plays a principal role in the assembly and maintenance of the 26S proteasome (Imai et al., 2003), indirectly HSP90 involved in protein degradation. Therefore, this mechanism also could possibly associate with the absence of changes in protein carbonyl in the present study when compared to pre exercise level for all trials.

5.6 CONCLUSION

In conclusion, the data obtained in this study showed that the acute consumption of quercetin significantly increased the level of plasma quercetin however this does not affect the physiological response to exercise, thermotolerance and plasma antioxidant capacity as well as heat shock response during exercise heat stress. This finding also suggested that the increase of eHSP70 and eHSP90α might act as supplementary antioxidant as

there are no changes in protein carbonyl level throughout the exercise trials, therefore the increase of heat shock protein may provide protection from oxidative damage during exercise heat stress.

CHAPTER 6

Study 3: Effects of Acute Antioxidant Supplementation on Extracellular (Plasma) and Intracellular (Muscle and PBMC) Heat Shock Protein (HSP70) Post 2 Days in Response to Exercise Heat Stress.

6.1 INTRODUCTION

It has been shown that an exercise stimulus and extreme environmental conditions such as heat stress sufficient to induce the increase of HSPs extracellularly (plasma and serum) (De Maio, 2011, 2014; Lee et al., 2017; Ogura et al., 2008; Périard et al., 2012; Ruell et al., 2014; Whitham & Fortes, 2008; Whitham et al., 2006). In support, the previous chapter (**Chapter 5; Study 2**) demonstrated that there was a noticeable increase in plasma concentration of HSP70 during exercise heat stress in all trials regardless of the supplementation consumed. Therefore the results indicated that acute and repeated intake of quercetin might not have effects on the acute response of extracellular HSP during exercise heat stress. HSPs can be expressed intracellularly or extracellularly as a circulating (plasma) protein (Bittencourt & Porto, 2017; Ghazanfarp & Talebi, 2013b; Henstridge et al., 2016; Kregel, 2002).

Exercise induces the release of eHSP72 in the systemic circulation; however, this finding was not followed by elevation of HSP72 in contracting muscle (iHSP72) (Walsh et al., 2001). mRNA increases immediately after acute exercise and remains high for a few hours, while protein content of HSP72 within the contracting skeletal muscle has been observed only hours or even days after exercise, thus iHSP72 expression might occur subsequently after increases of eHSP72 in the circulation (Walsh et al., 2001). A recent study (Lee et al. 2017) also discovered that eHSP72 levels corresponded to the periods of exercise heat stress and recovery however; the 15% increase in eHSP72 post-exercise disappeared 1 hour post-exercise, while the response of iHSP72 to exercise heat stress

remained elevated and high even after 24 hours recovery (~2.5-fold baseline values). In contrast to eHSP72, iHSP72 seems to continue to increase during the 24 hours period after exercise (PBMC; Périard et al. 2015), 48 hours post exercise and up to 7 days post exercise (muscle; Morton et al. 2006).

When cells are exposed to exercise and heat stress, HSPs are upregulated intracellularly and they are thought to involve with multiple cytoprotective functions, including molecular chaperones which is necessary in inhibiting the aggregation of folded protein, assisting the correct protein refolding and transferring the protein safely to the correct compartment (Lancaster & Febbraio 2007; Morton et al. 2006; Ghazanfarp & Talebi 2013). Intracellular HSPs (iHSPs) are expressed in variety of cells and organs (Henstridge et al., 2016) such as the heart (Chen et al., 2014; Salo et al., 1991; Skidmore et al., 1995), liver (Chen et al., 2014; Salo et al., 1991), brain (Walters et al., 1998), monocyte (Lee et al., 2014; Périard et al., 2015; Taylor et al., 2011), PBMC (Chang et al., 2010; Kuennen et al., 2011; Lee et al., 2017; Lovell et al., 2007; Zuhl et al., 2014) and most notably skeletal muscle (Khassaf et al., 2003; Khassaf et al., 2001; Morton et al., 2007; Morton et al., 2009; Puntschart et al., 1996). Under stress conditions, these proteins could be released from cells into the extracellular environment or enter the systemic circulation and may interact with a wide range of target cells (Calderwood et al. 2007).

Extracellular HSPs (eHSPs) have been suggested as a form of cellular messenger or may act as danger signal in response to the stress, injury, infection and cell damage (Fleshner & Johnson, 2005). eHSP70 has the ability to activate the innate immune response by binding to receptors on the damaged cell surface to protect them from subsequent insults (De Maio 2011; Jolesch et al. 2012; Borges et al. 2012). The detection of HSP70 in the blood of patients suffering from a variety of diseases indicate the importance of measuring

eHSP (De Maio, 2011). Furthermore, the presence of HSP70 in the blood has also been correlated with improved survival of critically ill patients (Pittet et al., 2002; Ziegler et al., 2005).

Additional data that supports an increase in intracellular and extracellular of HSPs during exercise is associated with the elevation of body temperature during exercise (Amorim et al., 2008; Gibson et al., 2014; Ogura et al., 2008; Périard et al., 2015; Périard et al., 2012). It has been suggested that there is a temperature threshold or a "minimum endogenous criteria" for induction of HSPs (Amorim et al., 2008).

If the concentration of eHSPs were thought to reflect a spillover of iHSPs concentrations, then the present study would expect to see similar trends in eHSP70 as those seen with iHSPs induced by exercise heat stress. Therefore, the purpose of this present study was to investigate the effect of acute quercetin supplementation on relationship between eHSP70 (plasma) and iHSP70 expression in PBMC and skeletal muscle samples pre and post 48 hours following exercise heat stress. It was hypothesised that the expression of eHSP70 and iHSP70 (PBMC and skeletal muscle) concentration would be similar in response to acute quercetin supplementation after exercise heat stress. This present study also determined the expression of muscle heat shock factor-1 (HSF-1) for all trials.

6.2 METHODS

6.2.1 Participants

Nine male participants were recruited in this study from the students of University of Bath. Their physical characteristics and physiological capacities of the participants were presented in **Table 6.1**.

Based on previous study which was carried out by (Khassaf et al. 2003) revealed that a total 9 participants are required to achieve 80% power to detect the effect of supplementation on intramuscular heat shock protein (HSPs) by using one-tailed t-test for two dependent means (matched pairs) with an alpha level of 0.05. Therefore a total of 12 will be recruited to account for an anticipated 20% drop-out associated with similar previous investigations.

Table 6.1 Physical characteristics and physiological capacities of the participants (Mean ± SD).

Parameters	Means ± SD
Age (years)	**22 ± 2**
Height (cm)	**1.77 ± 0.05**
Body mass (kg)	**71.6 ± 3.8**
Maximum oxygen uptake (ml.kg^{-1} min^{-1})	**50.3 ± 3.3**
Body mass index (BMI) (kg.m^{2})	**23 ± 1.5**
Running speed at 50% V̇O2max (km.h^{-1})	**8.2 ± 0.9**
Running speed at 70% V̇O2max (km.h^{-1})	**10.8 ± 0.9**

6.2.2 Procedures

Participants were requested to come to the laboratory on 11 occasions. The first two visits were for the preliminary tests, which included a maximum oxygen uptake ($\dot{V}O_2max$) test and familiarisation test. The following 3 visits were the experimental trials with 2 visits of muscle biopsy along with PBMC collection before each biopsy session (pre and post) during each trial.

6.2.3 Preliminary tests

Maximum oxygen uptake ($\dot{V}O_2max$) and familiarisation tests were carried out as described in the general method (**Chapter 3, section 3.6.1** and **3.6.2** respectively).

6.2.4 Muscle Biopsy

The study experimental protocol is shown in **Figure 6.1** and the protocol details are described as follows. Two days before started the main experimental trials, participants were required to attend the pre muscle biopsy session. Samples were obtained from the vastus lateralis muscle in the thigh under a local anaesthetic. The muscle biopsies took place at the University of Bath Laboratories. Once removed from the thigh, each muscle sample was immediately 'snap-frozen' by immersion in liquid nitrogen and stored at -80°C pending subsequent analysis for intracellular heat shock protein (HSP70 and HSC70 and HSF-1).

Two days following each exercise protocol, a further muscle biopsy sample was obtained from each participant. Each trial was separated by at least 7 days from post biopsy to another pre biopsy (Morton et al. 2006). For the second and third trials' the study protocol will be identical to the first trial.

6.2.5 Peripheral Blood Mononuclear Cells (PBMC) Collection & Separation

PBMC samples were collected on the same day as muscle biopsies were taken (pre and post 2 days exercise trial). Blood (12mL) was collected before muscle biopsies were taken. The method for PBMC samples collection was described in the general method (**Chapter 3, section 3.6.4**).

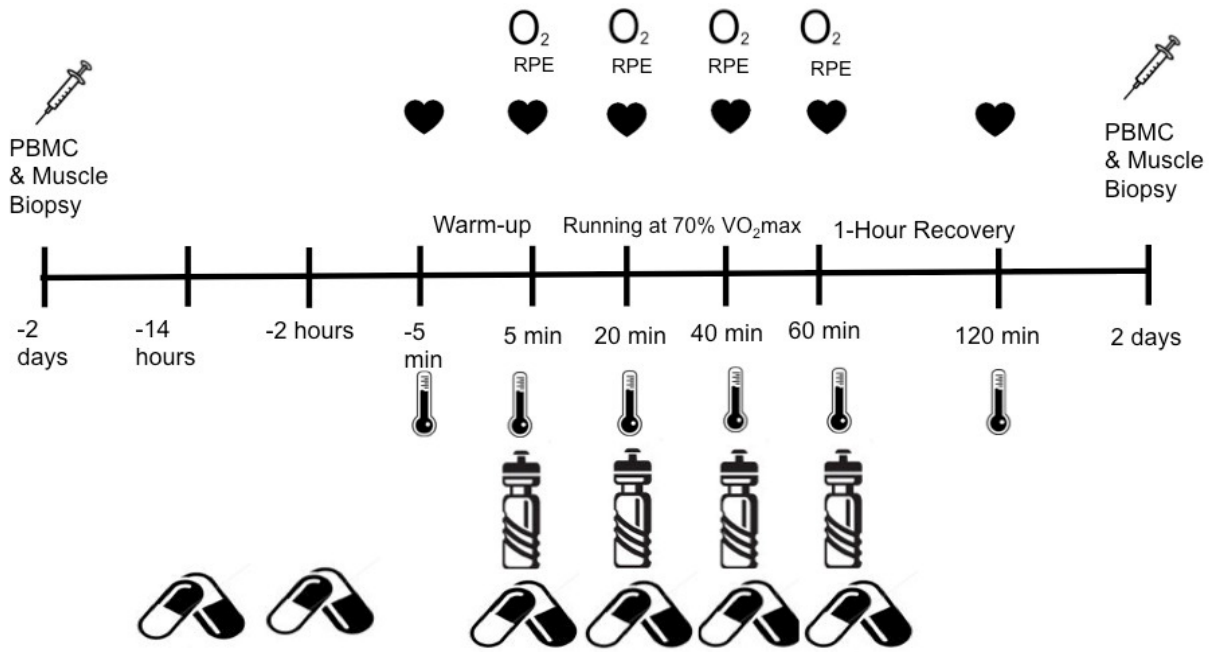
6.2.6 Experimental trials

Two days after muscle biopsies were taken, participants visited the lab for their first experimental trial between 08:30 and 9:00 following an overnight fast from 23:00. However, they were permitted to drink plain water. They were requested to consume 500 ml of plain water about one to two hour before exercise. They also were asked to abstain from alcohol; caffeine and refrain from strenuous exercise the preceding 48 hour.

All participants were randomly assigned, within crossover design, to complete three trials (Quercetin (Q), Quercetin plus vitamin C (QC), or placebo (P) with at least 14 days apart between trials. Participants were required to run for 60 min or until rectal temperature (Trec) reached 39.5°C. All exercise trials P, Q and QC took place in regulated environmental chamber; room temperature and relative humidity were well maintained as shown in **Table 6.2**. Absence of significant different in exercise intensity (**Table 6.2**) between trials may indicate that the intensity was well controlled.

Table 6.2 Environmental conditions and exercise intensity for all trials (Mean ± SD).

	Placebo	**Q**	**QC**	***P*-value between trials**
Room Temperature (°C)	32.3 ± 0.3	33.1 ± 0.3	33.4 ± 0.3	*p = 0.073*
Relative Humidity (%)	26.0 ± 2.0	28.7 ± 1.7	30.1 ± 1.7	*p = 0.331*
Intensity (70% $\dot{V}O_2$max)	73.4 ± 1.2	70.4 ± 1.3	70.3 ± 1.2	*p = 0.523*



RPE: Rating of perceived exertion

O_2 : Expired air collection

: Heart rate

: Blood sampling

: Rectal temperature

: Plain water ingestion

: Antioxidant supplementation

Figure 6.1 Study experimental protocol.

6.2.7 Muscle Samples Analysis

Preparation of muscle samples for Western blot analysis

The muscle samples were homogenised in protein homogenisation buffer containing 1% SDS (S/P530/53, Fisher Scientific), 20mM Tris pH 7.4 (T6066, Sigma) and protease inhibitors (S8820, Sigma) using a hand-held cordless homogeniser (VWR). The homogenised samples were placed on the end-to-end rotator for about one hour to solubilise the sample with the lysis buffer. Then the soluble fraction was separated from insoluble material by centrifugation of the homogenised samples at 13000 rpm for 10 minutes at room temperature in a bench-top centrifuge (Heraeus Biofuge 13).

To determine protein concentration, samples were diluted 1:10 with 0.1M NaOH and determined total protein concentration using Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific). The remaining muscle lysate was removed and placed into individual microcentrifuge tubes and stored at –80°C until further analysis. The muscle lysates were diluted with SDS sample buffer (70 mM Tris pH 6.8, 2% SDS, 0.01% bromophenol blue, 10% glycerol) supplemented with dithiothreitol (DTT), heated for 5 minutes at 95°C in dry-heating block (Techne, Dri-Block, DB-2A). The samples were electrophoresed in a gel electrophoresis apparatus (CBS, VWR) at an equal protein concentration of 40ug per lane together with 8µL of molecular weight markers (LC5800, Novex®, ThermoFisher) on a 10% tris-glycine SDS-polyacrylamide gel. The gels were transferred to a nitrocellulose membrane (BioTrace™ NT, Pall) using a semi-dry electrotransfer system (Bio-Rad). After transfer, the nitrocellulose membrane was rinsed in distilled water before staining with a small amount of 0.1% Ponceau S in 3% TCA (trichloracetic acid) for rapid reversible detection of protein bands on nitrocellulose membrane.

6.2.8 Peripheral Blood Mononuclear Cells (PBMC) Sample Analysis

Preparation of PBMC samples for Western blot analysis

Collections of PBMC samples were kept in Cryovial (Nalgene, Sigma-Aldrich). The vial was thawed in water bath at 37°C. The cells were aspirated carefully using a Pasteur pipette and then the cells were released carefully at the very bottom of a 15mL conical centrifuge tubes. After that, 10mL of warm media (RPMI-1640, Sigma Aldrich) were added to the cells carefully for washing step and spun at 300 x g for 7 minutes.

Following washing step, the media were pouring off carefully by leaving the pellet at the bottom of the tube. Then, 0.3µL of Benzonase (Benzonase® Nuclease HC, Merck, Denmark) was added directly to the pellet. The tube was shaken gently and left for 5 minutes. This purpose of this step is to reduce the viscosity of the samples caused by the excess DNA released from the cells.

After that, 50µL of SDS sample buffer (70 mM Tris pH 6.8, 2% SDS, 0.01% bromophenol blue, 10% glycerol) supplemented with dithiothreitol (DTT) were added to the tube. Then, the lysate samples were transfer to different Eppendorf tube and heated for 5 minutes at 95°C in dry-heating block (Techne, Dri-Block, DB-2A). The samples were electrophoresed in a gel electrophoresis apparatus (CBS, VWR) at an equal volume of 30µL per lane together with 8µL of molecular weight markers (LC5800, Novex®, ThermoFisher) on a 10% tris-glycine SDS-polyacrylamide gel. As the protein content of PBMC were very low, the present study decided to load equal volume of PBMC and to normalise to loading control (GAPDH). The gels were transferred to a nitrocellulose membrane (BioTrace™ NT, Pall) using a semi-dry electrotransfer system (Bio-Rad). After transfer, the nitrocellulose membrane was rinsed in distilled water before staining with a small amount of 0.1%

Ponceau S in 3% TCA (trichloracetic acid) for rapid reversible detection of protein bands on nitrocellulose membrane.

6.2.9 Western blot assay for muscle and PBMC samples (HSP70, HSC70 and HSF-1)

Samples were analysed for expression of stress proteins using standard Western blotting techniques. After blocking the nitrocellulose membranes in blocking solution consisting of 5% (w/v) dried skimmed milk powder (Marvel) in Tris-buffered saline (TBS-T, 0.9% NaCl, 10 mM Tris pH 7.4, 0.1% Tween 20) at room temperature for 60 min with gentle rocking. The membranes were incubated overnight with following primary antibodies (1:1000 dilution in TBS-T supplemented with 1% bovine serum albumin): anti-HSP70/HSP72 (SPA-8134-F; Enzo Life Sciences), anti-HSC70 (ALX-804-067-R050; Enzo Life Sciences), anti-HSF-1 (ADI-SPA-950-D: Enzo Life Sciences), anti-Actin (A2066, Sigma), anti-GAPDH (60004-1-Ig, Proteintech).

After overnight incubation, the membranes were washed 6 times with TBS-T for 5 minutes each before the final 60-minute incubation in secondary antibody at the required dilution (anti Rabbit IgG-HRP conjugate 1:2000 dilution and anti-Mouse IgG-HRP conjugate 1:2000 dilution) in blocking solution. The membranes were washed again extensively before incubation with enhanced chemiluminescent substrate (ECL, GE Healthcare or SuperSigna West Dura Extended Duration Substrate, Thermo Scientific). Protein bands were visualised using chemiluminescent imager using chemiluminescent imager (Epi Chemi II Darkroom, UVP) and quantified with Vision Works LS Software (UVP). Samples from each participant were applied to the same gel and the content of HSPs were normalised to the loading control; Actin for muscle samples and GAPDH for PBMC samples.

6.2.10 Plasma Quercetin Analysis

Total plasma quercetin (quercetin and its primary metabolites) pre and post 2 days exercise trials were measured using liquid chromatography–tandem mass spectrometry as previously described (Wang & Morris 2005). Plasma quercetin analysis was carried out as described in the general methods (**Chapter 3, section 3.9.6**).

6.3 Data Analysis

All statistical analyses were performed using the IBM Statistical Package for Social Sciences (SPSS version 24). Descriptive statistics were calculated measure mean and standard deviation (SD). Two-way ANOVA with repeated measures was used to analyse all variables. Where sphericity was broken, P values were corrected for by using the Greenhouse-Geisser method. In order to investigate the relationship between the post 2 days expression of eHSP70 and both intracellular of HSP70 (mHSP70 and PBMC HSP70), as well as the relationship between the post 2 days expression of mHSP70 and mHSF-1, Pearson's correlation analysis was performed. All the statistical significance was accepted at $p \leq 0.05$. All data were expressed as means ± standard deviation (SD).

6.4 RESULTS

6.4.1 Plasma Quercetin

Figure 6.2 represents the concentration of plasma quercetin (ug/L) for each exercise trial P, Q and QC pre 2 days and post 2 days. No significant differences in term of time ($F(1,24)=0.059$; *p=0.810)* and interaction ($F(2,24)=0.496$; *p=0.615)* were found.

The means of plasma quercetin for P, Q and QC trial were 366.6 ± 81.3ug/L, 352.7 ± 81.3ug/L and 458.1 ± 81.3ug/L respectively. Even though there was no interaction between trials; the mean plasma quercetin of QC trial was slightly higher compared to P and Q trials.

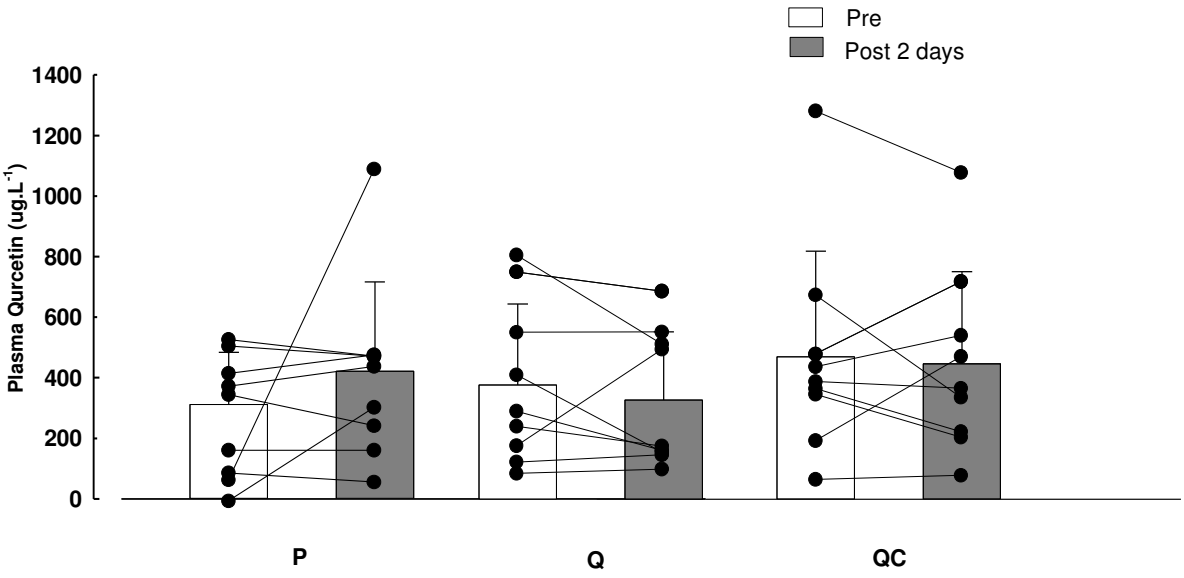


Figure 6.2 Plasma quercetin (ug/L) for pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC).

6.4.2 Extracellular HSP70

Figure 6.3 shows the concentration levels of HSP70 in plasma pre 2 days (baseline) and post 2 days in response to acute supplementation of quercetin during exercise heat stress. There were no significant effect in term of time (F(1,24)=0.618; *p=0.439)* and interaction between trials (F(2,24)=0.07; *p=0.832).* Mean plasma concentration of HSP70 for P, Q and QC trials were 1.796 ug.mL^{-1}, 1.856 ug.mL^{-1} and 2.252 ug.mL^{-1} respectively.

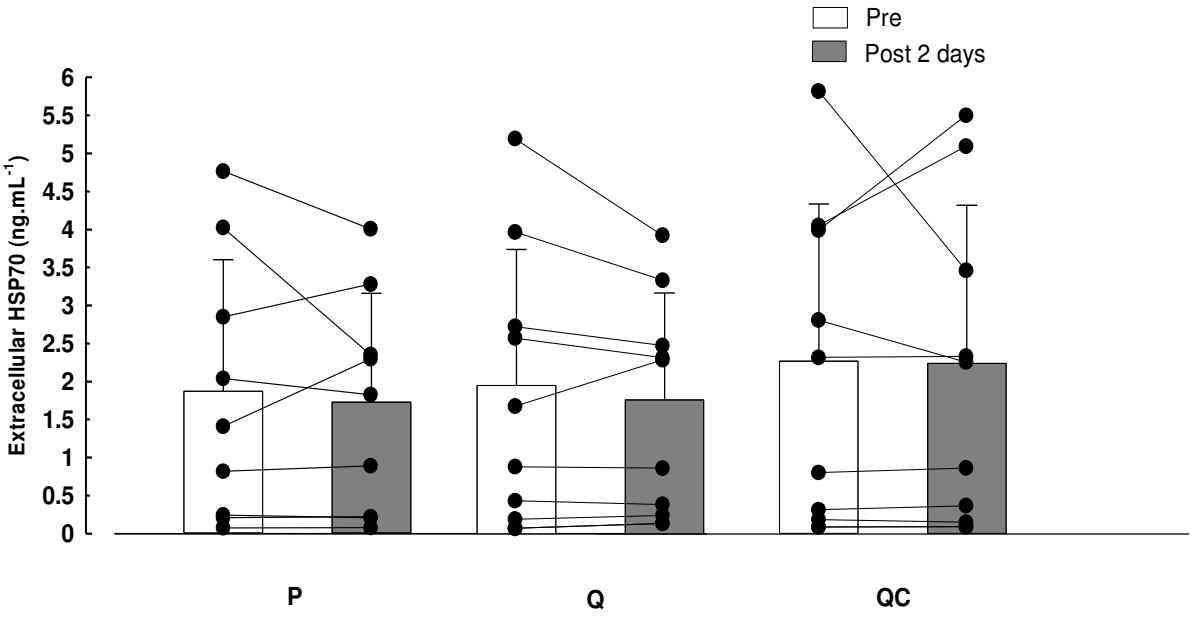


Figure 6.3 represents eHSP70 for pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC).

6.4.3 Muscle Heat Shock Protein 70 (mHSP70)

Figure 6.4 illustrates the changes in muscle expression of HSP70 at pre 2 days (baseline) and after 2 days in response to acute supplementation of quercetin during exercise heat stress. A two way ANOVA revealed there were no significant differences in term of time and interaction for mHSP70 F(1,24)=0.278; *p=0.603);* (F(2,24)=0.088; *p=0.916).*

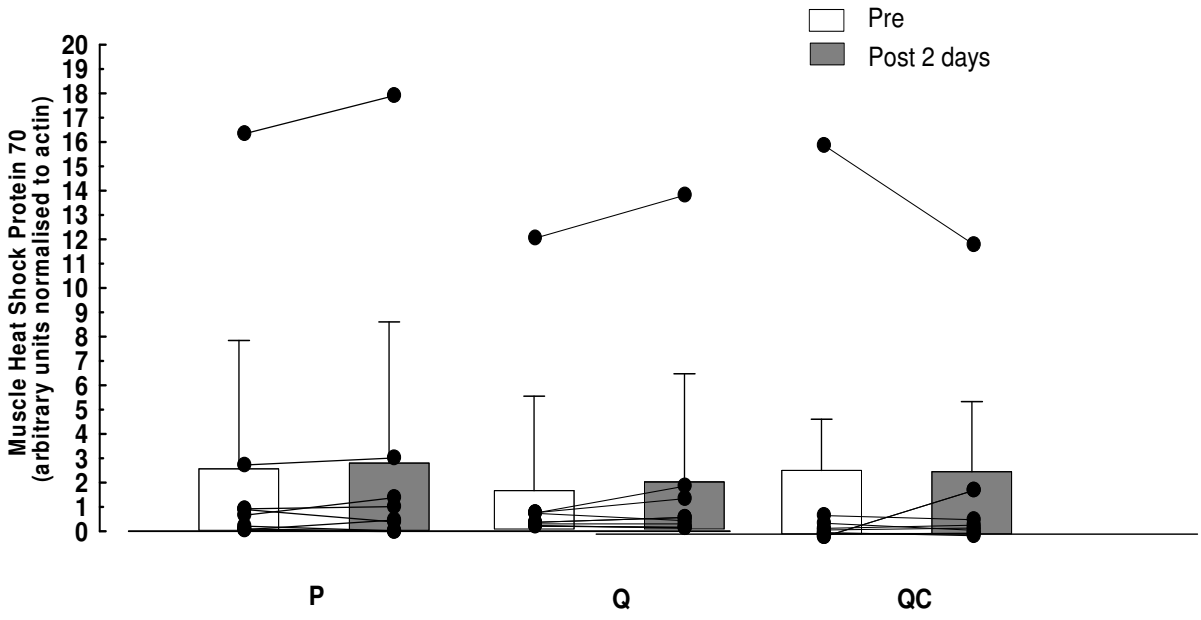
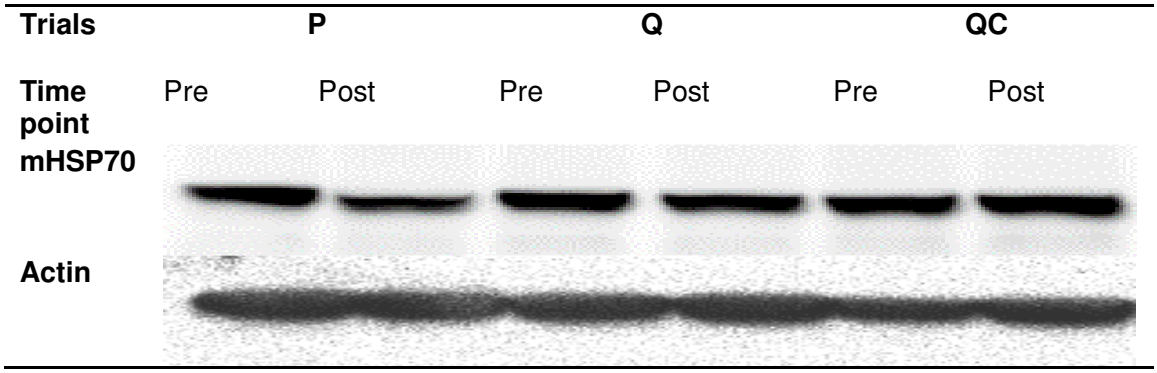


Figure 6.4 Relative skeletal muscles of HSP70 (mHSP70) a protein expression pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC) determined by Western blotting.

Figure 6.5 Representative images of Western blots for muscles HSP70 content responses to exercise from an individual subject chosen at random are also shown.



6.4.4 Muscle Heat Shock Cognate 70 (mHSC70)

There were no significant main effect of supplementation during exercise heat stress on muscle HSC70 between pre 2 days and post 2 days ($F(1, 24) = 0.854$, *p=0.365*) and the interaction ($F(2, 24) = 0.219$, *p=0.805*) for all trials. Based on the individual response (**Figure 6.6**), about 80% of the participants for each trial had no change between pre and post 2 days exercise heat stress. No interaction between trials may indicate that the acute supplementation have no effect on the expression of mHSC70 post 2 days.

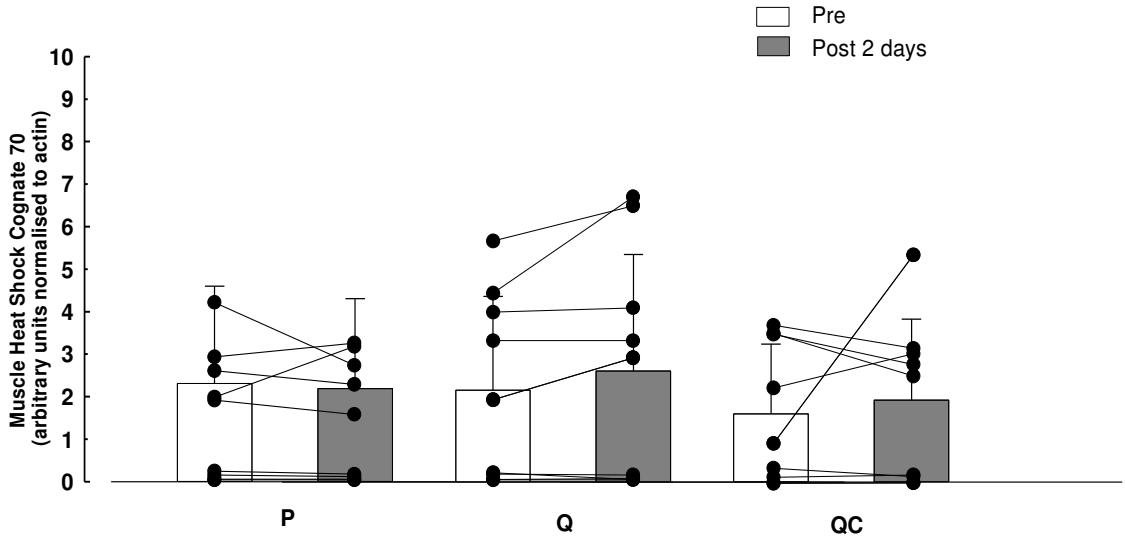
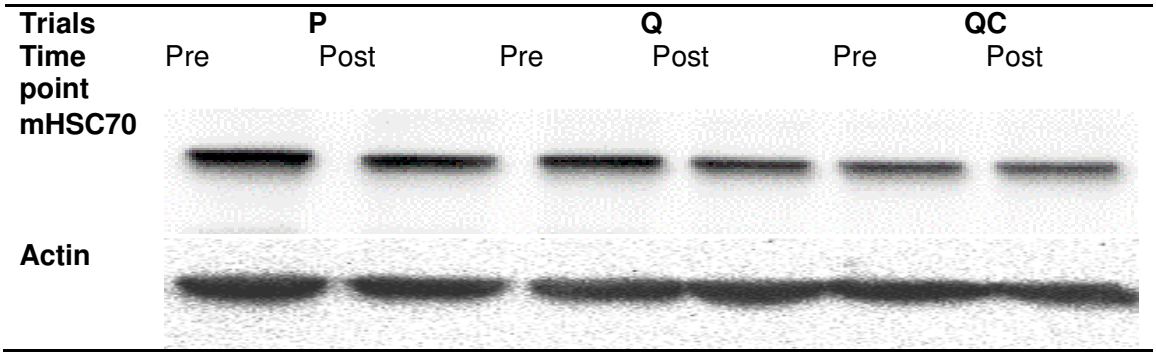


Figure 6.6 Relative skeletal muscles of HSC70 (mHSC70) a protein expression pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC) determined by Western blotting.

Figure 6.7 Representative images of western blots for muscles HSC70 content responses to exercise from an individual subject chosen at random are also shown.



6.4.5 Muscle Heat Shock Factor-1 (mHSF-1)

No observable significant different between pre and post 2 days in mHSF-1 in response to acute supplementation during exercise heat stress (F(1,24)=0.408; *p=0.529*) (**Figure 6.8**). In addition, no interaction between trials was found (F(2,24)=0.816; *p=0.454*).

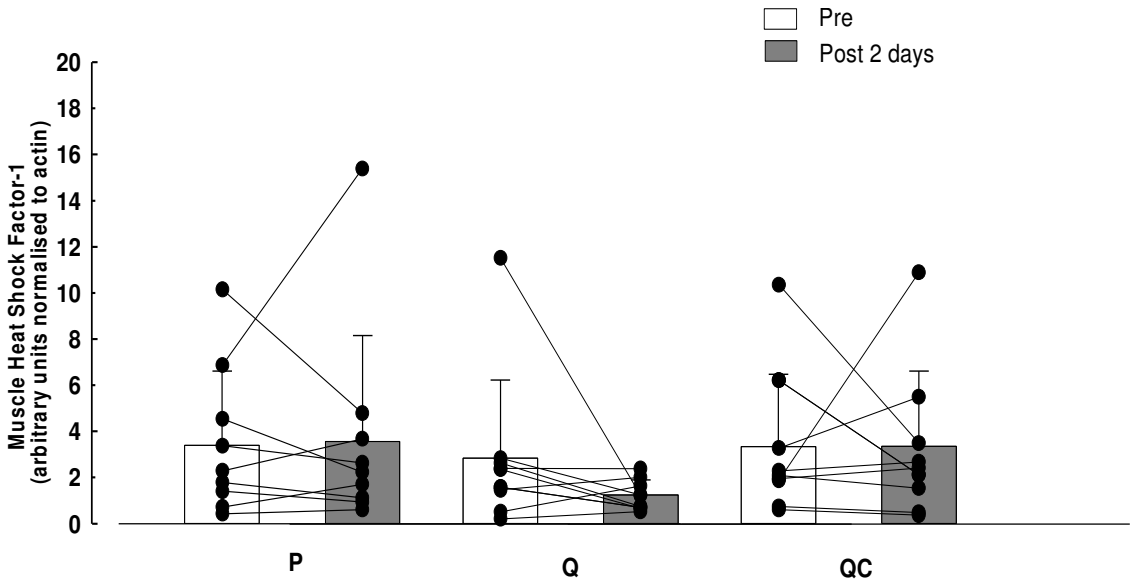
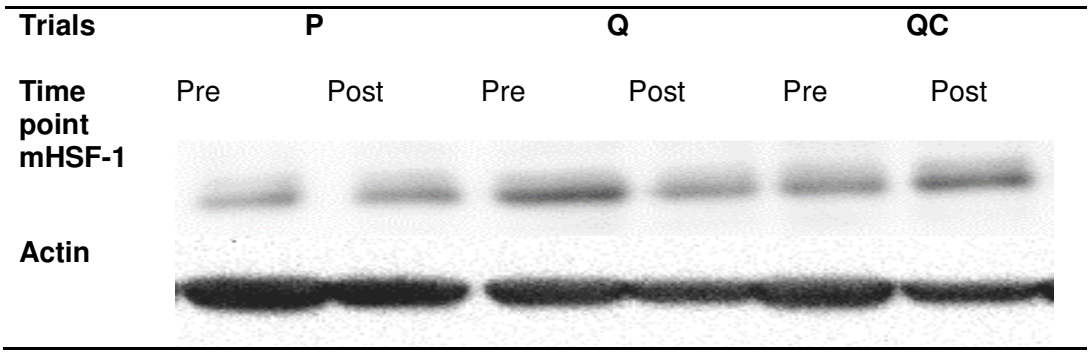


Figure 6.8 Relative skeletal muscles of HSF-1 (mHSF-1) a protein expression pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC) determined by Western blotting.

Figure 6.9 Representative images of western blots for muscles HSF-1 content responses to exercise from an individual subject chosen at random are also shown.



6.4.6 PBMC Heat Shock Protein (PBMC HSP70)

HSP70 expression in PBMC was not significantly different between baseline (pre 2 days) and post 2 days ($F(1,24)=0.001$; *p=0.974*). Furthermore, no significant different was detected between trials ($F(2,24)=0.081$; *p=0.923)*. Based on individual response (**Figure 6.10**), about 44% of participants in P trial show increased in the expression of PBMC HSP70. While, about 67% and 89% of participants in Q and QC trials show decreased or no change in the expression of PBMC HSP70, respectively.

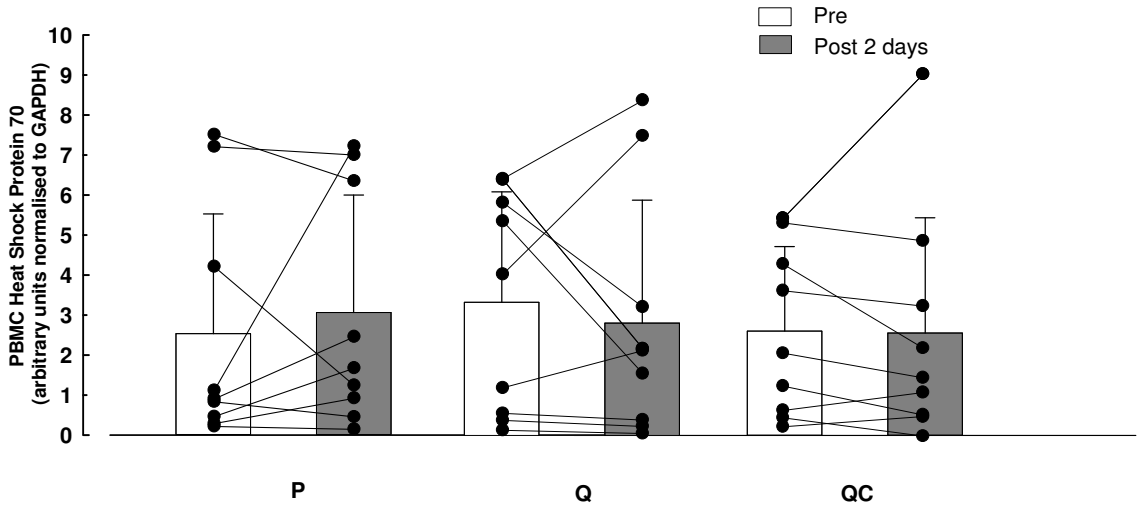
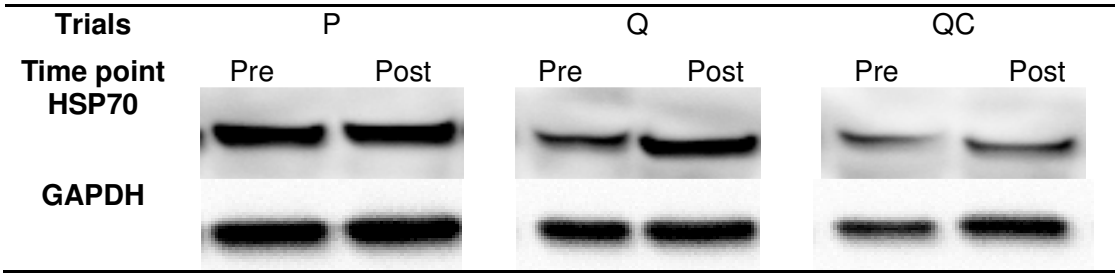


Figure 6.10 Relative peripheral blood mononuclear cell of HSP70 (PBMC HSP70) protein expression pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC) determined by Western blotting.

Figure 6.11 Representative images of western blots for PBMC HSP70 content responses to exercise from an individual subject chosen at random are also shown.



6.4.7 PBMC Heat Shock Cognate (PBMC HSC70)

The acute supplementation during exercise heat stress did not induce any difference from pre to post 2 days exercise (F(1,24)=0.001; *p=0.974)* in the expression of PBMC HSC70 and no significant difference from between trials was found (F(2,24)=0.081; *p=0.923)* (**Figure 6.12**).

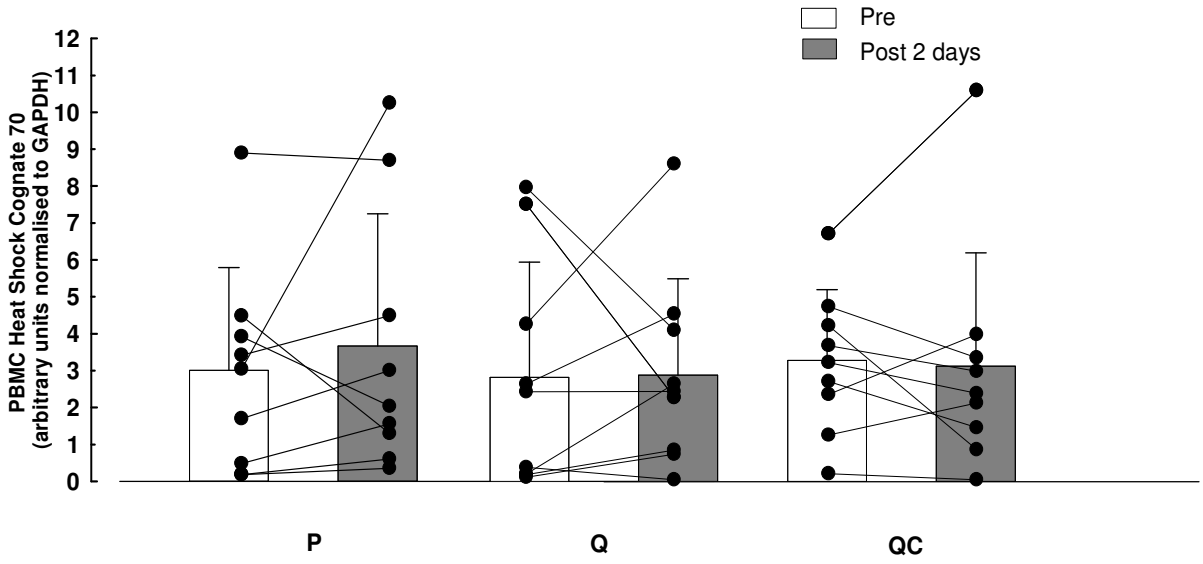
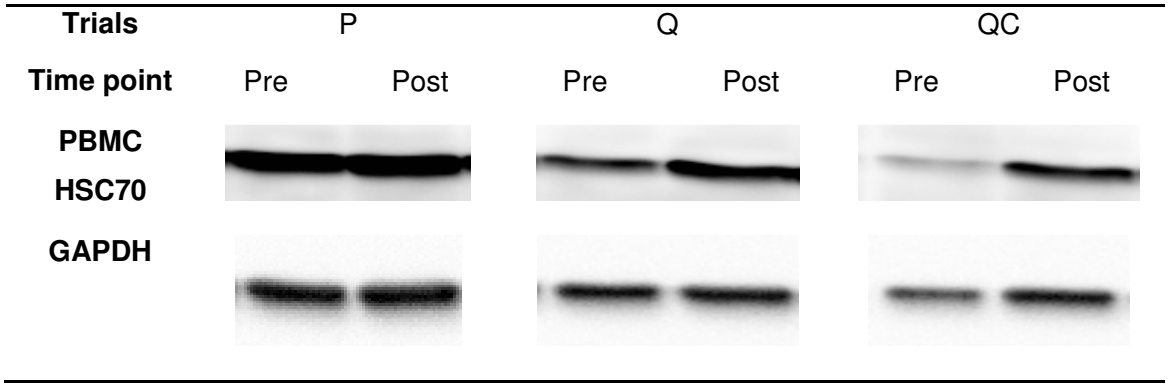


Figure 6.12 Relative peripheral blood mononuclear cell of HSC70 **(**PBMC HSC70) protein expression pre and post 2 days for all trials: Placebo (P); Quercetin (Q) and Quercetin+Vitamin C (QC) determined by Western blotting.

Figure 6.13 Representative images of western blots for PBMC HSC70 content responses to exercise from an individual subject chosen at random are also shown.



6.4.8 Relationship between eHSP70, mHSP70 and PBMC HSP70

Scatterplot showing that (**Figure 6.14, A**) there was no relationship between post 2 days protein expression of mHSP70 and eHSP70 for all trials (r = -0.424, r^2= 0.180, p<0.05). In addition no relationship (**Figure 6.14, B**) was detected between expression of PBMC HSP70 and eHSP70 for all trials (r = 0.068, r^2= 0.005, p= 0.736). These results suggest that muscle and PBMC might not be the tissue sources of HSP70 release into extracellular compartment.

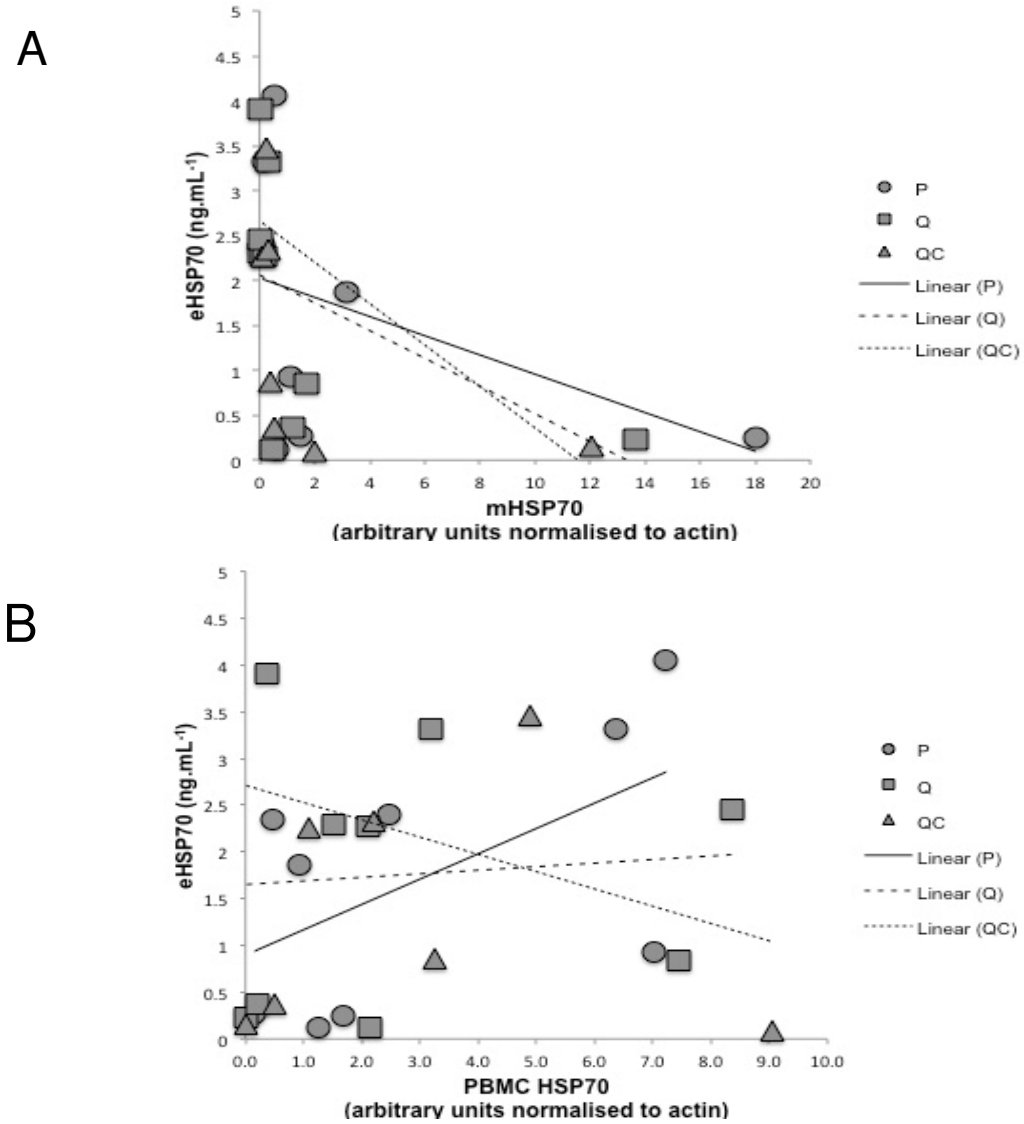
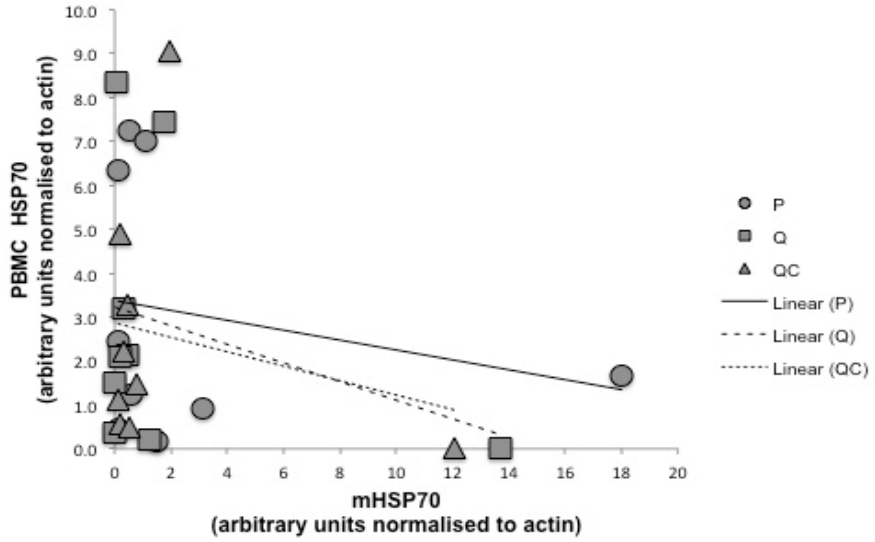


Figure 6.14 (A) Correlation between post 2 days protein expression of mHSP70 and eHSP70. **(B)** Correlation between post 2 days protein expression of PBMC HSP70 and eHSP70.

6.4.9 Relationship between mHSP70, PBMC HSP70 and mHSF-1

Scatterplot showing that (**Figure 6.15, A**) there was no relationship between post 2 days protein expression of mHSP70 and PBMC HSP70 for all trials (r = -0.238, r^2= 0.057, p=0.232). In addition, no relationship (**Figure 6.15, B**) was detected between expression of mHSP70 and mHSF-1 for all trials (r = -0.268, r^2= 0.072, p= 0.117). These results suggest that both tissues (muscle and PBMC) were independent to each other in expression of HSP70 post 2 days exercise.

A



B

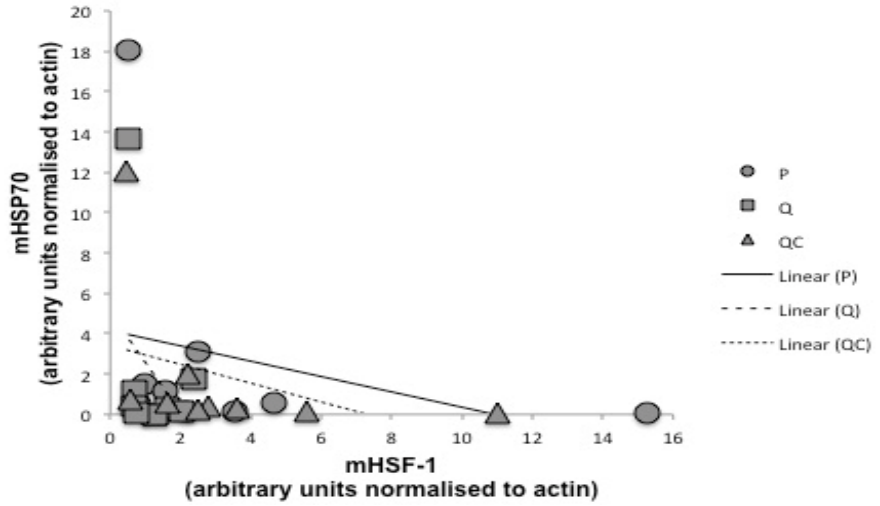


Figure 6.15 (A) Correlation between post 2 days protein expression of PBMC HSP70 and mHSP70. **(B)** Correlation between post 2 days protein expression of mHSP70 and mHSF-1.

6.5 DISCUSSION

The aim for the present study is to investigate the effect of acute quercetin supplementation on the relationship between the response of eHSP70 (plasma) and iHsp70 expression in skeletal muscle and PBMC samples pre and post 48 hours following exercise heat stress. The most notably finding in this study that the HSP70 protein expression in skeletal muscle and PBMC were not correlated with the plasma level of HSP70 (eHsps) at 48 hours post exercise. The absence of relationship between both iHSP70 (muscle and PBMC) and eHSP70 at 48 hours post exercise might imply that the contracting skeletal muscle and PBMC are not the tissue source of the exercise-induced expression in the eHSP70 concentration during recovery.

The aim of the present study was to demonstrate a positive relationship between both iHSP70 (muscle and PBMC) and eHSP70. We hypothesized that eHSP70 might be released from the stressed/damaged cells of any organ as the production of this protein requires transcriptional and translational steps in the cells (Asea, 2005; Liu et al., 2006). In support, previous study (Febbraio et al., 2002) revealed the eHSP72 from lysed muscle cells and intact muscle cells are not the primary source in releasing eHSP72 into the circulation, but this stressed muscle cells synthesised HSP72 in order to provide intracellular protection. Apart from muscle and PBMC, evidences suggested other organs such as liver (Febbraio et al., 2002) and brain (Lancaster et al., 2004) have the ability to synthesise HSP70 and being responsible to export this protein into blood circulation. The release of eHSP70 involves various mechanisms before it is seen in the systemic circulation and could originate from numerous tissues and cell types. The absence of a relationship between both iHSP70 (muscle and PBMC) and eHSP70 indicate that muscle and PBMC might not be the tissue sources of HSP70 release into the extracellular compartment.

Intracellular HSP70 (such as mHSP70 and PBMC HSP70) acts as a chaperone and cytoprotective agent by providing protection and restoring normal functions of the damaged cells, following injury after stress and if the stress occurred subsequently (Ghazanfarp & Talebi, 2013a; Kregel, 2002; Lancaster & Febbraio, 2007; Locke, 1997). Therefore, functions of eHSP70 are perhaps distinct from the cytoprotective functions of iHSP70; these support the dual role of HSPs, which are reliant on their location of expression (intracellular vs. extracellular). This eHSP70 acutely released into the circulation, activates the immune response, which is necessary for the body's defence system to fight infection and inflammation (Basu et al., 2000; Campisi et al., 2003; Hightower & Guidon, 1989); and to allow the system to return to basal levels when the stress ceases.

The present study demonstrated that the acute supplementation of quercetin might not give any effects on post 48 hours eHSP70 response induced by exercise heat stress as no changes were found between trials. The possible reason could be that the eHSP70 might return to basal levels when the stress ends (Calderwood et al., 2007; De Maio, 2011; Jolesch et al., 2012). As shown in a previous study (Magalhães et al., 2010), the heat stress (40°C; relative humidity 45%) increased eHSP72 from baseline to post exercise and then returned to resting values 1 hour post exercise. In contrast to eHSP72, iHSP72 seems to continue to increase during the 24 hours period after exercise (PBMC; Périard et al. 2015), 48 hours post exercise and up to 7 days post exercise (muscle; Morton et al. 2006).

Human PBMC are frequently used to investigate the response of HSP70 with exercise heat stress (Fehrenbach et al., 2001; Hunter-Lavin et al., 2004; Magalhães et al., 2010; Périard et al., 2015) as these cells respond promptly compared with skeletal muscle which

may take 48 hours post exercise to respond (Morton et al., 2006). Furthermore, Lancaster & Febbraio (2005) demonstrated that PBMCs that have been exposed to heat shock increased exosomal HSP72, which indicated that PBMCs have the ability to actively release HSP72. However, the exercise heat stress conducted in the present study did not induce statistically significant up regulation of the investigated PBMC HSP70 from pre to post 48 hours of exercise for both (Q and QC) supplemented trials. The absence of changes in PBMC HSP70 could be due to HSP70 return to baseline as 48 hour post exercise is too long for these cells to continue to increase; post 24 hours could be more appropriate time to detect the increased of PBMC HSP70 (Périard et al., 2015).

The response of HSP70 in the skeletal muscle (Khassaf et al., 2001; Morton et al., 2006; Thompson et al., 2001) demonstrated that peak expression of HSP70 occurred post 48 hours of exercise, however this study demonstrated that the HSP70 expression in skeletal muscle and PBMC were completely blunted as there were no significant differences detected between pre and post 48 hours of exercise for all trials. A number of studies have revealed that quercetin decreases the heat-induced synthesis of HSPs by inhibiting the up-regulation of HSF-1 (Hansen et al., 1997; Hosokawa et al., 1990; Hosokawa et al., 1992). However, as this present study failed to demonstrate any difference from supplemented trial compared to placebo, it is implausible to conclude that quercetin successfully blunted the response of mHSP70 and PBMC HSP70 post 48 hours exercise heat stress in Q and QC trials.

A relationship was not discovered between mHSF-1 and mHSP70, however this study could not detect any HSF-1 in PBMC samples through western blotting. The heat stress condition in this present study might induce the disassociation of HSF-1 from HSP70/HSC70 and then from this phase, quercetin might act as inhibitor by inhibit the

HSF-1 activation, thus down regulated the new HSPs synthesis (Nagai et al., 1995). Based on this speculation, this present study expects to observe negative relationship between mHSF-1 and mHSP70, however no relationship was found between mHSF-1 and mHSP70.

Despite the absence of a significance difference in plasma quercetin pre and post 2 days of exercise, the fact that quercetin concentration was successfully maintained during the exercise heat stress (**Study 2, Chapter 5, Figure 5.6**) verified that the quercetin exists in the participants bloodstream. The level of plasma quercetin post 48 hours in QC trial was slightly higher than P trial. However, the ability of quercetin to restrain the elevation of mHSP70, PBMC HSP70 and eHSP70 protein expression up to 48 hours post exercise remains unclear due to absence of significant interaction in the expression of both iHSP70 (muscle and PBMC) and eHSP70 compared to P trial.

6.6 CONCLUSION

In conclusion, the data obtained from this present study demonstrated that there is no positive relationship between both intracellular of HSP70 (muscle and PBMC) and plasma HSP70 (eHSP70) post 2 days in response to acute antioxidant supplementation during exercise heat stress. Thus, the results indicate that the release of eHSP70 could originate from other tissue or cells. Additionally, absence of a difference between trials in the expression of mHSP70 and PBMC HSP70 as well as plasma concentration of HSP70 suggests that quercetin does not play a role as a HSP70 inhibitor in plasma, muscle and PBMC post 48 hours. In addition, the large variations observed among individuals in the plasma quercetin analysed serves to support the idea that in order to study the effect of antioxidant in both iHSPs and eHSPs, it is necessary to restrict food intake containing flavonoid among participants.

CHAPTER 7

GENERAL DISCUSSION

Throughout the studies conducted in this thesis, the central aim was to investigate the influence of hyperthermia and antioxidant supplementation on redox balance and heat shock protein response to exercise. **Chapter 4** explored the redox balance responses of military recruits who either had confirmed EHI compared to controls who did not suffer from EHI, during intense and exhaustive military training. **Chapter 5** investigated the effects of acute antioxidant supplementation on redox balance and extracellular heat shock response during exercise heat stress. **Chapter 6** addressed the question of whether the acute antioxidant supplementation would induce similar trends in extracellular heat shock response that are seen in the intracellular heat shock response after exercise heat stress.

A summary of the results of all four experimental chapters is outlined below as follows:-

Study 1 (Chapter 4)

Aim: To explore the redox balance in military recruits with confirmed EHI compared to controls who did not suffer from EHI after exhaustive military training.

Key Findings:

- One of the most notable findings in the present study is that the plasma antioxidant capacity was significantly elevated by both LM and LR events for heat illness and CON groups.
- However lipid peroxides and protein carbonyl did not change for heat illness and CON groups during both LM and LR events.

Study 2 (Chapter 5)

Aim: To examine whether the acute intake of antioxidant would minimise the effects of oxidative stress and reduce the heat shock response during exercise heat stress.

Key Findings:

- Exercise heat stress significantly elevated plasma antioxidant capacity (*p<0.001),* eHSP72 (*p=0.009*) and eHSP90 (*p<0.001*) immediately post exercise and post 1-h exercise when compared to pre exercise, but supplementation did not affect the change.
- Plasma quercetin significantly increased across time during exercise in Q and QC trials (*p=0.02*). Even though there was no significant interaction between trials, a trend toward significance (p=0.16) was detected. Based on **Figure 5.6** the level of plasma quercetin in P trial was about half of the level of plasma quercetin in QC trial. Absence of significant interaction between trials could be due to large variation of plasma quercetin was observed among the participants.
- No changes in protein carbonyl immediately post exercise and post 1-h exercise when compared to pre exercise, and no significant interaction between trials were detected.

Study 3 (Chapter 6)

Aim: To investigate whether the acute antioxidant supplementation would induce similar trends in extracellular heat shock response those are seen in the intracellular heat shock response after exercise heat stress.

Key Findings:

- The effect of acute antioxidant supplementation during exercise heat stress on skeletal muscle, PBMC and plasma HSP70 (eHSP70) was not observed as there was no interaction effect between trials.
- Therefore, it is implausible to conclude regarding the potential of quercetin as an inhibitor of the expression of HSP70 in muscle and PBMC as well as eHSP70.

- Additionally, there were no positive relationships observed between both iHSP70 (muscle and PBMC) and eHSP70. This suggests that eHSP70 could be released from others tissue or cells.

7.1 Effects of hyperthermia on redox balance.

Several studies examined that the heat stress alone or heat stress combined with exercise heat stress could cause oxidative stress in humans during exercise (Laitano et al., 2010; McAnulty et al., 2005; Morton et al., 2007; Ohtsuka et al., 1994; Quindry et al., 2013; Sureda et al., 2015). Thus, heat stress could be a supplementary factor that induces oxidative damage to DNA, proteins and lipids (Bruskov et al., 2002; Grasso et al., 2003; Zhao et al., 2006).

There is a possible linkage between oxidative stress and heat, which convincingly propose that oxidative stress could be a crucial adverse factor in boosting the severity of heat illnesses (Adachi et al., 2009). Finding in **Study 1 (Chapter 4)** suggested that the noticeable increase in plasma antioxidant capacity for both EHI casualties and CON during LM and LR events could be mediated by induction of the endogenous antioxidant release such as uric acid (Aguiló et al., 2005; González, 2008; Hellsten et al., 1997) and ascorbic acid (Gleeson et al., 1987), thus increased in plasma antioxidant capacity may have contributed in attenuating the plasma lipid peroxidation and protein carbonyl level as no changes in lipid peroxides and protein carbonyl were detected from pre to post events.

Another possible justification, it could be that the participants in **Study 1** who are Parachute Regiment Trainees (Para) from British Army, which is generally know that military duties mainly involved in endurance training that has been claimed to reduce lipid peroxidation by augmenting the body's defence capabilities (Ginsburg et al., 1996; Sureda

et al., 2015; Yagi, 1992). Regular training is known to decrease the accumulation of oxidative protein and DNA damage as well as heighten the resistance against ROS induced lipid peroxidation (Radak et al. 2001). This implied that antioxidant defence system with regular training might reduce the lipid peroxide level and the damage caused by free radicals (Alessio et al., 2000; Nojima et al., 2008). Therefore, the lack oxidative damage evidence could be a consequence of their nature due to habitual endurance training and their well-trained status.

Lacking in oxidative stress could also involve the second defence mechanisms, which are heat-shock proteins (HSPs) as a response to heat stress. These proteins are also reported to have an antioxidant effect (Fehrenbach & Northoff, 2001). As evident in **Study 2** (**Chapter 5**), exercise heat stress significantly elevated eHSP70 ($p=0.009$) and eHSP90 ($p<0.001$) throughout the exercise trials with no changes were detected in protein carbonyl across time. Finding in **Study 2** also revealed that exercise heat stress significantly increased plasma antioxidant capacity. Therefore, the absence of changes in protein carbonyl suggested that it is likely due to the protective effects of antioxidant properties of eHSPs or induction of the endogenous antioxidant release as evident by the increased of plasma antioxidant capacity.

Furthermore, the possible mechanism for the clearance of protein carbonyl group could be facilitated by the 20S proteasome system alongside with the excretion through urine and protein uptake by the active muscle during exercise (Wadley et al., 2016). The 26S proteasome is an integral part of the cell's mechanism to degrade proteins, Hsp90 found to be interacts with the 26S proteasome. Therefore, eHSP90 significantly increased throughout the exercise trial, could plays a principal role in the assembly and maintenance of the 26S proteasome (Imai et al., 2003), indirectly HSP90 involved in protein

degradation. Therefore, this mechanism also could possibly associate with the absence of changes in protein carbonyl.

Therefore both studies revealed that the thermal strain encountered by physically active participants did not induce significant oxidative damage but stimulated the adaptive mechanisms including endogenous antioxidant and heat shock response to better tolerate the heat stress.

7.2 Effects of acute antioxidant supplementation on redox balance.

Since 1970's even until now, there are more than 150 studies investigating the effects of antioxidant supplementation to reduce oxidative stress (Peternelj & Coombes, 2011). Antioxidant supplementation may aid in protecting from cellular oxidative damage by maintaining the redox balance and assisting in recovery by boosting the immune function after intense exercise, thus improve athletic performance (Close et al., 2016; Tauler et al., 2002; Tauler et al., 2003, 2008)

However, questions have been raised whether the body's natural antioxidant defense system is sufficient to counterbalance the increase in FR with exercise or whether additional supplements are required. Interestingly, literature has emerged controversial issue about FR act as signalling molecules to stimulate antioxidant enzyme synthesis during exercise that leads to favourable exercise induced adaptations (Ji et al., 2006; Radak et al., 2014) but antioxidant supplementation could hamper these adaptations (Gomez-Cabrera et al., 2009). However, not all investigations revealed that antioxidant supplementation hampers exercise-induced activation of redox sensitive signalling pathways (Petersen et al., 2012).

Clinical trials in human demonstrated that after ingestion of a 250-500 mg quercetin, it can be rapidly increased in plasma within 15-30 minutes, its peak concentration reached at approximately 120-180 minutes, started to decrease around 360 minutes of ingestion and returned to baseline levels after 24 hours (Davis et al., 2009)**.** Thus, the study design in **Study 2** which to consume the supplements 14 hours, 2 hours and every 20 minutes during exercise trial are to maintain the bioavailability of the antioxidant in the blood. **Study 2** demonstrated that the study design successfully increased and maintained the level of quercetin in the blood during the trial. Therefore, with acute and repeated quercetin supplementation, human could achieve a considerable level of quercetin in plasma (Hollman et al. 1997; Manach et al. 2005).

In addition, the mixed supplementation of quercein and vitamin C was designed in the **Study 2** to improve the bioavailability and bioactive effects of quercetin as suggested by previous researchers simultaneous ingestion of quercetin with vitamin C, folate, and additional flavonoids improves bioavailability of the quercetin (Harwood et al., 2007; Manach et al., 2005; Moon & Morris, 2007). Therefore, an adequate plasma ascorbate level therefore should be maintained when high doses of quercetin are supplemented (Boots et al., 2008). Consistent with the literature, **Study 2** found that the level of plasma quercetin in QC trial was double the level of plasma quercetin in P trial. Since there was a very slight trend toward significance ($p=0.16$) was detected, this study believed that the acute intake of quercetin together with vitamin C enhanced the bioavailability of quercetin.

However, the increased level of plasma quercetin did not affect the level of protein carbonyl and plasma antioxidant capacity as no interaction was detected between trials. The increase in plasma antioxidant capacity in **Study 2** presumably portray the release of urate and ascorbate into the blood during exercise (Aguiló et al., 2005; Yanai & Morimoto,

2004). Furthermore, the absence of changes in protein carbonyl might due to the upregulation of heat shock proteins (HSPs), as eHSPs have been found to increase in the plasma after exercise heat stress for all trials.

One possible reason for the lacking of the effect of Q or QC on the oxidative stress might be due to no flavonoids-based food restriction were performed during the study period (such as blueberries, strawberries, apples, celery, oranges or quercetin in particular), however all the participants were asked to refrain from consume any mineral or vitamin supplement (other than those provided), or any other antioxidant supplements for 2 weeks before and during the trials. Therefore, this would be one of the study limitations as consuming food contained of flavonoid might affects the level of plasma quercetin. Therefore, the finding in **Study 2** believed, it could be more effective in future study if all the participants were refrained from taking any flavonoid-based foods throughout the trials.

7.3 Effects of acute antioxidant supplementation on heat shock response.

There is very little published research about the effects of acute applications of quercetin on heat shock response caused by exercise heat stress in humans. A number of studies have exposed that quercetin decrease the heat-induced synthesis of HSPs (Hansen et al., 1997; Hosokawa et al., 1990; Hosokawa et al., 1992; Kuennen et al., 2011). This mechanism involved suppressing heat shock factor-1 (HSF-1) DNA-binding ability by affecting its conformational changes which occurs in the cells (Hosokawa et al., 1992; Kuennen et al., 2011; Nagai et al., 1995), consequently it would affected the process of transcription and protein translation by HSF-1 (**Figure 7.1**), eventually prevent the induction of new HSP70 in response to exercise heat stress.

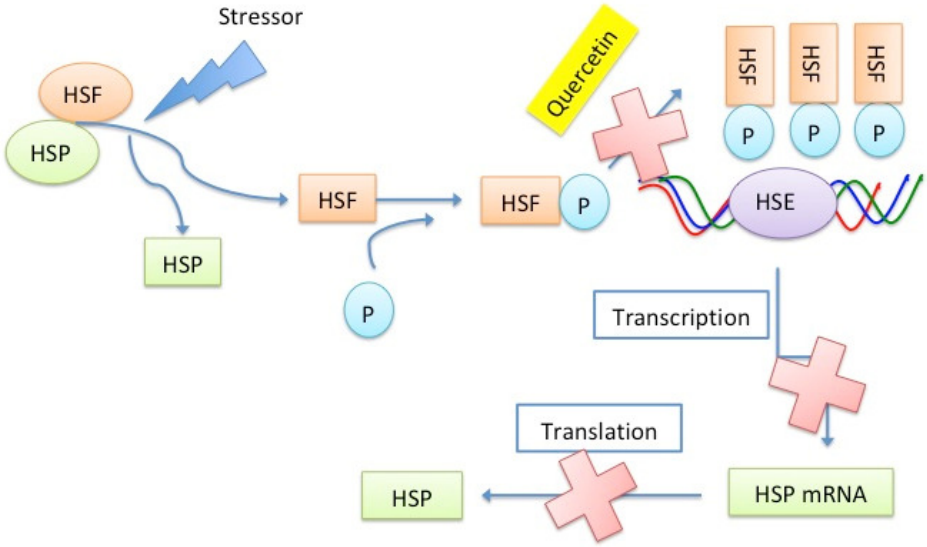


Figure 7.1 Proposed schematic diagram of the mechanism of Quercetin inhibits the upregulation of HSP70.

Therefore, the idea of **Study 3** which acute intake of antioxidant supplements would reduce up regulation of cellular HSPs in response to heat exposure and the response would be similar in eHSP70. However, finding in **Study 3** failed to demonstrate that these acute intakes of quercetin have the ability to inhibit the upregulation of iHSPs (muscle and PBMC) and eHSPs response as no difference was found pre and post 2 days exercise heat stress. In contrast to **Study 3**, ingestion of quercetin (2000mg/day) for a week found to inhibit the heat-induced of HSP70 in PBMC post, 2-hour post and 4-hour post exercise when compared to pre-exercise level (Kuennen et al., 2011). In vivo investigations demonstrated that applications of quercetin successfully verified as a hyperthermia sensitizer in thermotherapy against cancer by suppressing the HSP70 increases (Asea et al., 2009; He et al., 2012; Whitesell et al., 2009; Yang et al., 2011). Therefore, it is unclear whether the acute, high and repeated dose of Q and QC ingestion could prevent the up-regulation of mHSP70 and PBMC HSP70 as well as plasma concentration of HSP70 as there is no interaction when compared to P trial.

In addition, absence of positive relationship between both intracellular of HSP70 (muscle and PBMC) and plasma HSP70 (eHSP70) post 2 days in response to acute antioxidant supplementation during exercise heat stress might indicate that releasing of eHSP70 could originate from others tissue or cells and in this context of present study, muscle and PBMC might not be the tissue sources of HSP70 release into extracellular compartment post 2 days exercise heat stress (Bittencourt & Porto, 2017; Febbraio et al., 2002; Febbraio et al., 2002; Lancaster et al., 2004).

7.4 Limitations

There are some limitations in the experimental studies in this thesis.

In **Chapter 4**, the post-event blood sample were not fasting blood, therefore the results from the post-event blood sample might be influenced by what they had eaten during the breakfast. Also, the results in the present study were not corrected to plasma volume changes. The present study would have been more convincing if exact plasma volume changes have been measured, then these should allow more informed interpretation of the true significance of observed changes in the biomarkers involved

In **Chapter 5**, and **Chapter 6** the lack of significance interaction effect between trials might be due to no flavonoid type food restriction were performed during the study period (not allowed to consume food rich in flavonoids such as blueberries, strawberries, apples, celery, oranges or quercetin in particular), Therefore, consuming food contained of flavonoid might affects the level of plasma quercetin among the participants in throughout the study conducted.

In addition, study in **Chapter 6** could not measure the protein expression of mHSF-1 in PBMC through western blotting. Therefore, it is difficult to extrapolate whether the acute intake of quercetin or quercetin + vitamin C successfully suppressed the induction of HSF-1 in PBMC.

7.5 Future direction.

Further investigation need to be carried out.

- Following findings from **Study 1** (**Chapter 4**), further investigation needs to be carried out as EHI is associated with hyperthermia which is believed to increased oxidative stress, therefore considering oxidative stress as a confirmatory of heat illness use remains unclear, but its worthy of further investigation by analysing more biomarkers (e.g uric acid, vitamin C, antioxidant enzymes and ratio of reduced glutathione to oxidised glutathione (GSH/GSSG)) to measure oxidative stress in order to confirm the occurrence of oxidative stress during heat illness.
- Following findings from **Study 2** (**Chapter 5**), further investigations with a longer supplementation period (e.g 7 days) and analysing more biomarkers (e.g uric acid, vitamin C, antioxidant enzymes and ratio of reduced glutathione to oxidised glutathione (GSH/GSSG)) to measure oxidative stress in order to confirm the occurrence of oxidative stress during exercise heat stress as an ideal biomarker to quantify oxidative stress does not exist. The results will be very valuable since to date there was no other study investigating effect of quercetin on the occurrence of oxidative stress in response to exercise heat stress.
- Following findings from **Study 3** (**Chapter 6**), further investigations is also encouraged with longer supplementation period (e.g 7 days) and adding more post-exercise samples of plasma muscle and PBMC as stress response is typically

observed several hours post-exercise. The results will be very valuable since to date there was no other study investigating effect of quercetin on intracellular and extracellular heat shock protein in response to exercise heat stress.

7.6 Conclusion

The results from this thesis emphasise that both environmental heat stress and associated hyperthermia could potentially influence the human redox balance during exercise. Besides, there is reasonable evidence that acute quercetin co-ingestion with vitamin C has the potential to improve the bioavailability and bioactive effects of quercetin, however the effects of quercetin supplementation in reducing oxidative stress in response to exercise heat stress remains to be elucidated. In addition, the ability of acute ingestion of quercetin could provide protective effects due to its antioxidative properties to suppress the intracellular and extracellular heat shock response remains uncertain and worthy for further investigation.

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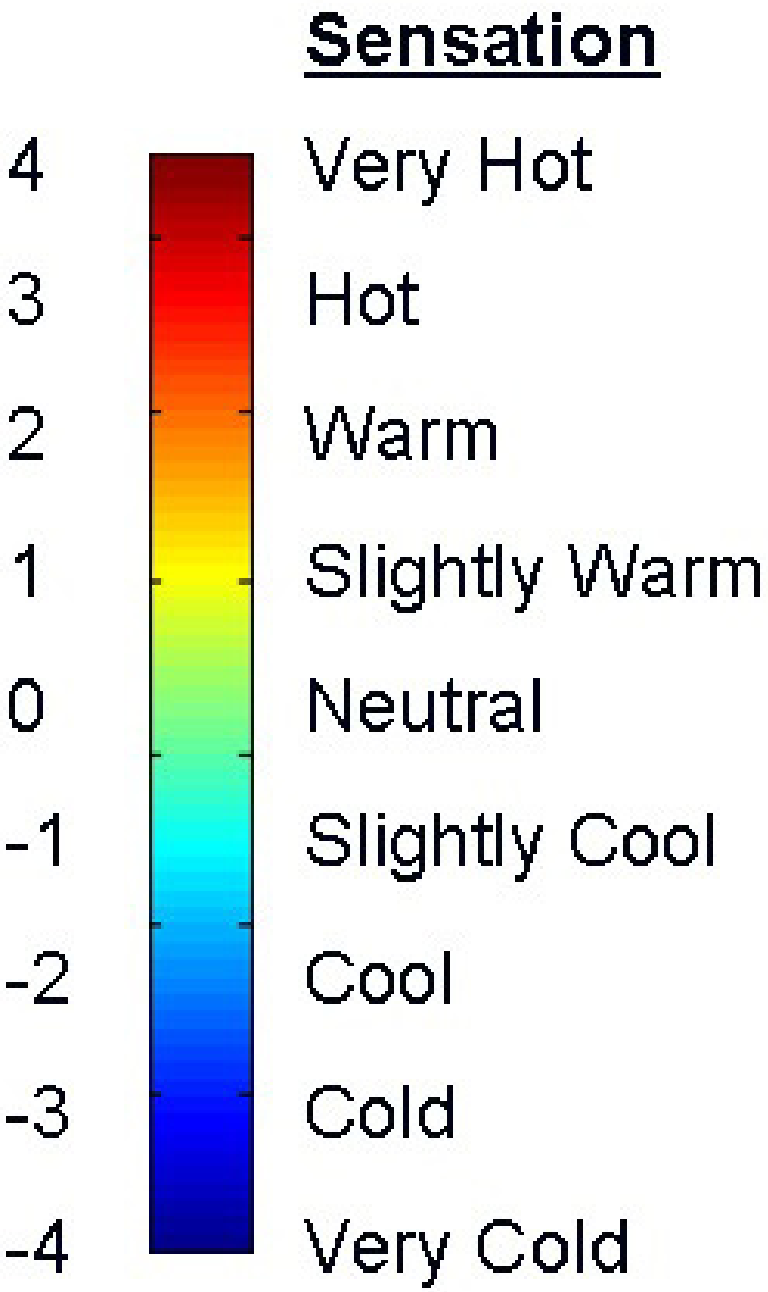
Rating	Perceived Exertion
6	No exertion
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion


Sensation
4
Very Hot
3
Hot
2
Warm
1
Slightly Warm
0
Neutral
-1
Slightly Cool
-2
Cool
-3
Cold
-4
Very Cold